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The Editor comments

A CASE OF SUPPLY AND DEMAND

Business and financial experts emphasize the delicate balance which exists in our economy between the supply of goods and services as compared with the demand for them. When inventories of raw materials such as steel, supplies of finished products such as automobiles, or the available labor force for employment, drop below or exceed certain norms, the economy immediately reacts. We then find ourselves in a period of either recession or inflation. Often, variations of as little as a few per cent are sufficient to trigger such a reaction.

Similar forces are involved in the dissemination of scientific literature. If adequate publication facilities are not available, some meritorious reports might not find their way into the literature. Conversely, if publication facilities exceed the supply of good papers, the standards of journal quality are bound to be seriously jeopardized.

It has come to our attention that a sister organization is considering the launching of a new publication in the field of medicinal chemistry. While we recognize the great importance of adequate media for the publication of such research reports, it is our conviction that the establishment of another journal in this field would be most ill-advised at this time.

Currently available pharmaceutical research periodicals, including THIS JOURNAL, have not reached full publication capacity. Present limitations are in the quantity of suitable manuscripts rather than in the journal space in which to publish them. Consequently, a new periodical in this field can only serve to reduce the number of worthy papers available to any given journal. The result will be detrimental to the quality of all of the publications involved, and therefore, this matter merits our deep individual interest and concern.

We bring this information to the attention of our readers in order that they might express themselves in an informed manner. Such expression should convince prospective publishers that adequate scientific coverage would best be achieved by further improvement in our present journals rather than the issuance of additional publications.

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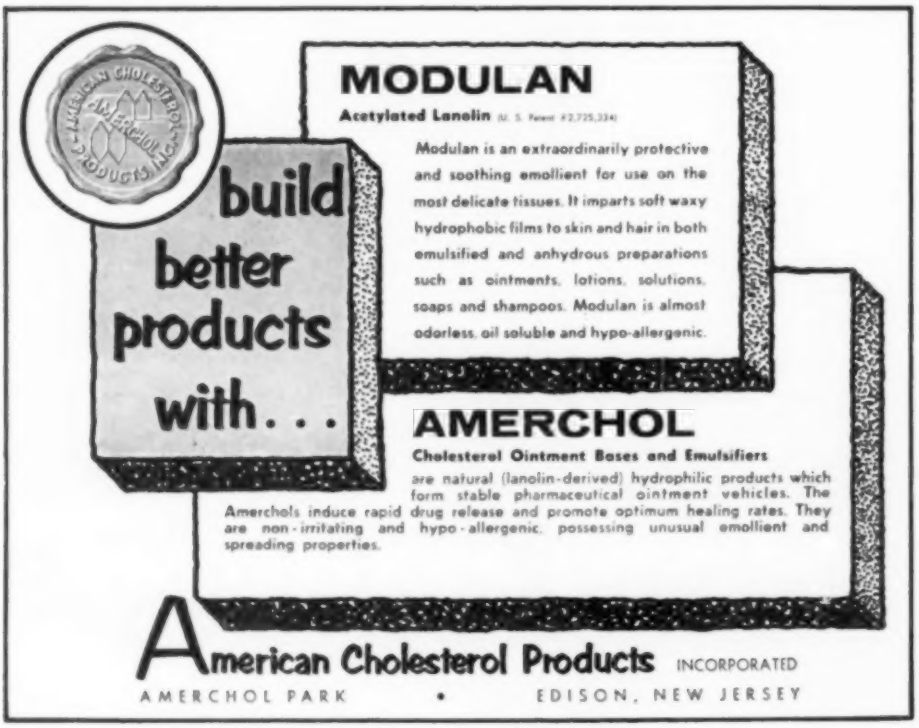
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Review Article

Kinetics of Drug Absorption, Distribution, Metabolism, and Excretion

By EINO NELSON

THE kinetic aspects of drug absorption, distribution, metabolism, and excretion have not been previously reviewed in the English language. Two discussions or reviews on the subject have been included in German publications. One of these constituted one issue of *Die Pharmazie* (1) and was prepared by Druckrey and Kupfmuller. The other is included in a book written by Dost (2). Dost's bibliography on kinetic and related topics is extensive. More than two decades ago, Teorell published two papers in the English language which were a theoretical treatment of the kinetic consideration under discussion here (3). Some of the literature existing on the subject at that time, as well as literature on kinetics of some physiological substances, was briefly reviewed in these papers. Relatively recently, Robertson published an extensive review on the theory and use of isotopes in the determination transfer rates (4). In Robertson's review, work with physiological substances was of primary interest (4). The same is true in the rather extensive papers published by Russell (5) and by Odeblad, Westin, and Englund (6). Although not concerned with drugs, many fundamental principles of elimination and distribution are covered in a book chapter by Dominguez (7).

The present review is mainly concerned with drugs or substances normally foreign to the body although, since in many cases physiological substances are handled by the body, kinetically speaking, in the same manner as drugs, some of these cases are also considered. This review is further restricted to rate processes in the intact animal except where surgical removal of certain organs, e. g., nephrectomy, was a useful procedure to investigate kinetics and disposition. It should be pointed out that, while concentration-time relationships of drug in various body depots may in some cases be well predicted by a kinetic approach based on the determination of necessary rate constants and other parameters, such relationships do not necessarily delineate the pharmacological activity-time relationships. However, very little quantitative information is available on the last mentioned subject and a knowledge of concentration-time relationships is necessary to obtain these quantitative data.

GENERAL CONSIDERATIONS

The disposition of several drugs might be depicted by the following scheme (assuming oral ingestion of the drug at zero time):

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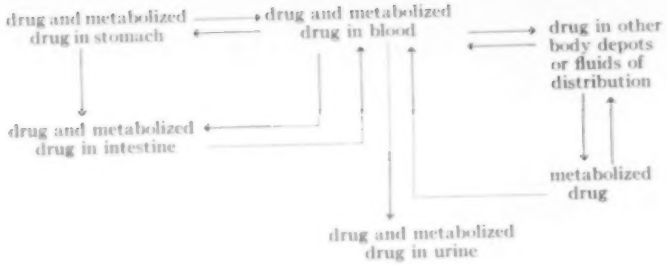

drug and metabolized drug in stomach
drug and metabolized drug in blood
drug in other body depots or fluids of distribution
drug and metabolized drug in intestine
metabolized drug
drug and metabolized drug in urine

The above scheme is complex, but actually much less complex than that which actually exists in the intact animal. For example, transfer processes shown by a single line in the scheme consist, in most cases, of a series of processes. Other excretory or disposition processes may exist, successive modifications may be accomplished on the parent drug during its metabolism, etc. However, drug excretion or metabolism, for example, may often be describable by relatively simple kinetics. That this is so, results from several factors. Many of the processes shown, although in operation at all times while drug is in the body, involve only small amounts in comparison to the amount in fluid on which measurements are made. Biliary excretion occurs with many drugs and reabsorption of drug so excreted may take place from the intestine. This results in an enterohepatic circulation of drug, but unless the amount involved in this cycle is large, its influence on the concentration *vs.* time course of drug in blood will not be noticeable.

Another factor that may allow a complex scheme of processes to be described by relatively single mathematical expressions is the rapid equilibration of drug after its introduction into the body among the fluids in which it becomes distributed. For example, following intravenous injection, the kinetics of the distribution and elimination of many drugs may be described by the following scheme:

As will be seen later, a two-term exponential expression delineates the concentration *vs.* time course of drug in blood at all times it can be measured. However, within a short time after injection, drug in blood is in equilibrium with drug in the other fluids of distribution and kinetically the disappearance of drug from blood will be described by a single-term exponential expression for the bulk of the time drug is in the body. The length of time elimination is describable by the single-term expression depends on the relative value of the rate constants for the processes shown.

With increasing complexity in the scheme chosen to describe a drug's disposition, increasingly more complex mathematical expressions result from integration of the differential expressions that describe the rate of transfer or modification of drug. However, since the constants in the mathematical expressions must be evaluated from the experimental data and since the mathematical expressions have no power of discrimination in themselves, the fact that the expression with constants evaluated does fit the experimental data may mislead one into believing the model chosen is absolutely correct. Thus, a kinetic approach to study drug elimination and modification must be made with due recognition of this situation.

Error in analytical methods applied to biological fluids is usually inherently high. Thus, the effect of unknown processes involving measurable amounts of drug may be hidden in the experimental error of the assay method used for the drug.

An examination of the literature on the kinetics of absorption, distribution, etc., indicates that in the bulk of the cases transfer, elimination, and metabolism rates in intact animals are directly proportional to the concentration or amount at the site from which these processes take place. In the language of the chemical kineticist, these processes are "first order" or "monomolecular." In the metabolism of some drugs, processes appear to proceed at a constant rate independent of concentration. The analogy to chemical kinetics here is the "zero order" process. Unless otherwise stated, processes under discussion in this review will be first order.

Most drugs introduced into the blood become reversibly distributed in other body fluids or tissues. The sites at which these transfers take place are referred to as "compartments," "body

pools," or as the "other fluids of distribution." The latter designation will be most used here and will usually denote a site to which drug passes without specification as to whether or not this is extravascular fluid, selective binding by some particular tissues, etc. Kinetic analyses may indicate that more than one pool or compartment other than blood must exist to account for drug distribution. The kinetic analysis by itself cannot necessarily establish the reality of such additional compartments available to participate in distribution.

While consideration of the kinetics of the metabolism of drugs is not easily separable from other kinetic aspects when considering the latter, the literature may conveniently be divided into two groups, one of which has been mainly concerned with the kinetics of metabolism and the other with one or more of the other topics considered in this review. This review is, therefore, divided into two parts with the kinetics of drug metabolism being separated from the other topics, even though some overlapping inevitably results.

KINETICS OF DRUG ABSORPTION, DISTRIBUTION, AND EXCRETION

The first extensive studies on the kinetics of drug absorption and elimination appear to have been conducted by Widmark (8, 9). Using acetone as an indifferent narcotic, he studied the appearance and disappearance of this material from the blood after administration by several routes and appearance in expired air. He recognized that the material was both metabolized and excreted in the urine as well as eliminated by the lungs, and that these processes were competitive with each other. He found that after absorption of the dose the concentration decreased in an exponential fashion and that rate of elimination in expired air was directly proportional to concentration in the blood.

Later Dominguez (10–15), using creatinine and xylose as examples, pointed out in considerable detail the relationship between plasma concentration and excretion rate and the significance of other parameters involved in the elimination and distribution of these materials. The theoretical relationships experimentally verified by Dominguez are summarized in the following: when Cp was the concentration of material in the plasma at any time in any convenient units and when drug in plasma was in equilibrium with drug in other fluids of distribution, the rate of disappearance of drug from plasma, dCp/dt, was equal to

$$-\frac{dCp}{dt} = KCp \qquad \text{(Eq. 1)}$$

where the rate constant K was the elimination constant in reciprocal time. The constant K was the sum of the rate constants for the individual processes that were concerned with elimination and with the materials he studied these were two, namely, urinary excretion and utilization or metabolism. Then $K = k_1 + k_2$ where k_1 is the rate constant for urinary excretion and k_2 is the same quantity for metabolism. Both k's also have dimensions of reciprocal times. In general, if disappearance rate is given by Eq. 1, then K is the sum of all individual rate constants concerned with elimination. Integration of Eq. 1 gives

$$Cp = Cp^0 \exp[-Kt] \qquad \text{(Eq. 2)}$$

where Cp^0 was the plasma concentration at zero time. Alternatively, since drug in plasma was in equilibrium with drug in other fluids of distribution and since $CpVd = Ab$, where Vd and Ab were, respectively, the volume of distribution and amount in the body, Eq. 2 may be written as

$$Ab = Ab^0 \exp[-Kt] \qquad \text{(Eq. 3)}$$

where Ab^0 was the amount in the body at zero time.

For creatinine and xylose, excretion rate was directly proportional to plasma concentration. That is

$$dAe/dt = k_1 VdCp \qquad \text{(Eq. 4)}$$

where Ae was the amount excreted in any time and Vd the apparent volume of distribution of drug. After the substitution of Eq. 2 in 4, integrating the resulting expression, and evaluating the constant of integration at zero time under the condition that no excretion has occurred

$$Ae = \frac{k_1 VdCp^0}{K}(1 - \exp[-Kt]) \qquad \text{(Eq. 5)}$$

At infinite time $Ae = Ae^0$ where Ae^0 is the amount of a dose in circulation in the body eventually excreted in urine and

$$Ae = k_1 VdCp^0/K = \frac{k_1}{K} Ab^0$$

or

$$Ae^0/Ab^0 = k_1/K = f \qquad \text{(Eq 6)}$$

where f is the fraction of dose originally in circulation that was eventually excreted in the urine. Making use of the identity from Eq. 6, $k_1 = Kf$, in Eq. 4 gives

$$dAe/dt = KfVdCp \qquad \text{(Eq 7)}$$

and hence volume of distribution can be calculated by simultaneous measurement of excretion rate, plasma concentration, and knowledge of the value of the elimination constant from plasma, as

well as the fraction of a dose excreted unchanged in the urine.

It is of interest to point out that Eq. 5 may be written as

$$Ae = f\,Ab^0(1 - \exp[-Kt]) \qquad \text{(Eq. 8)}$$

and when excretion rate dAe/dt is plotted on semilogarithmic paper and a straight line results, the rate constant K for elimination from the body is found from the slope instead of the rate constant for excretion [*cf.* Stern (55, 56), Hough, *et al.* (57), Swintosky (16), and Hough (17)]. This follows since $dAe/dt = Kf\,Ab^0\,(\exp[-Kt])$
and

$$\log_e (dAe/dt) = -Kt + \log_e Kf\,Ab^0 \qquad \text{(Eq. 9)}$$

Kidney clearance as defined by the physiologist may be expressed in the terminology used here. Clearance, C, is defined as (18)

$$C = UV/P \qquad \text{(Eq. 10)}$$

where P is plasma concentration in mg./cc., U is urine concentration in mg./cc., and V is urine flow in cc./min. Then since $UV = dAe/dt = Kf\,Vd\,Cp$, from Eq. 7,

$$C = Kf\,Vd = k_1 Vd \qquad \text{(Eq. 11)}$$

Clearance from kidney function tests is usually determined with substances that are almost completely eliminated in urine so $f = 1$ and

$$C = K\,Vd \qquad \text{(Eq. 12)}$$

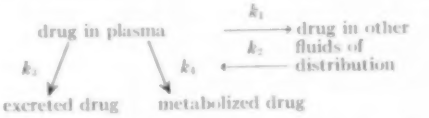
Although Dominguez did not use the term half life, $t_{1/2}$, this term is widely used now and is usually referred to as the biological half life of a drug. It is easily found from Eq. 2 to be

$$t_{1/2} = 0.693/K \qquad \text{(Eq. 13)}$$

Thus, it bears a distinct relationship to physiological processes determining the value of K and its use is incorrect, for example, to define duration of action when a drug has been incorporated into a dosage form to prolong action.

The preceding summary of the relationships presented by Dominguez and co-workers (10–15) are not in the terminology of the original publications. Furthermore, he defined an excretion constant, A, that was the product of k_1 and Vd (Eq. 4). Likewise, his utilization constant, B, is equivalent to the product of k_2 and Vd. The preceding summary did not introduce terms used by Dominguez to account for preadministration plasma levels or excretion rate of the material under study. This level and rate are the "blanks."

Later studies by Dominguez, *et. al.*, included measurements and their interpretations in the per-equilibrative phases of distribution following intravenous administration (19–21). The situation studied may be portrayed by the following scheme where an arrow indicates a process whose rate is directly proportional to amount present at the indicated sites:



In the scheme shown, plasma level is described by a two-term exponential expression of the form

$$Cp = A\exp[-\alpha t] + B\exp[-\beta t] \qquad \text{(Eq. 14)}$$

The constants A, B, α, and β are relatively complex functions of the rate constants for the processes shown in the preceding model as follows:

$$\alpha = \frac{b + \sqrt{b^2 - 4c}}{2}$$

$$\beta = \frac{b - \sqrt{b^2 - 4c}}{2}$$

$$b = k_1 + k_2 + K$$

$$c = k_2 K$$

$$K = k_3 + k_4$$

$$A = \frac{\alpha(Cp^0 k_1 Vp + \beta f\,Ab^0)}{k\,Vp\,(\beta - \alpha)}$$

$$B = Cp^0 - A$$

where Cp^0 is the plasma concentration at zero time, Ab^0 the dose of drug administered, f the fraction of the dose eventually excreted, and Vp the plasma volume. The ratio of the rate constants k_2/k_1 is the ratio of plasma volume to apparent volume of the other fluids of distribution.

The method of deducing the numerical values of the various constants has been described (21). Equilibrium between drug in plasma and drug in other fluids of distribution was rapidly reached with substances studied by Dominguez and his co-workers (creatinine, mannitol, galactose, xylose); hence, within a relatively short period of time plasma level was described by Eq. 2 with constants evaluated.

Volume of distribution may be calculated by

$$Vd = \text{dose}/Cp^0 \qquad \text{(Eq. 15)}$$

where Cp^0 is the extrapolated concentration at zero time from a semilogarithmic plot of plasma concentration *vs.* time following intravenous administration of drug. Calculation of this quantity by Eq. 15 is only an approximation and the accuracy depends on the rate of attainment of equilibrium between drug in plasma and drug in other fluids of distribution, as well as on the

amount eliminated during the attainment of equilibrium. Dominguez, Corcoran, and Page have derived and presented expressions to determine volume of distribution from plasma-level measurements more accurately (21).

The disappearance of galactose after intravenous injection was another interesting case studied by Dominguez and Pomerene (22). Excretion rate was directly proportional to plasma concentration, but plasma concentration did not decrease exponentially. This observation suggested that the rate of utilization of galactose was not directly proportional to plasma concentration. An expression was derived to describe plasma concentration of galactose as a function of time.

Other work by Dominguez and Pomerene has been the development of a method to calculate absorption rate following oral ingestion of drug and the application of the method to creatinine (23). In the terminology used in this review, the equation developed was

$$\text{absorption rate} = Vd\frac{dCp}{dt} + K\ VdCp \quad \text{(Eq. 16)}$$

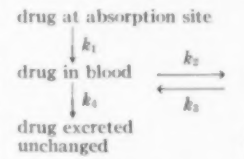
An application of the method of Dominguez and Pomerene to study the absorption rate of sulfaethylthiadiazole has been made by Swintosky and his co-workers (24).

The experimental work of Widmark, Dominguez, and others (25, 26) appears to have stimulated Teorell (3) to prepare a general theoretical treatment of the subject of the kinetics of distribution. His treatment of the subject is an extension of a less extensive, earlier treatment by Gehlen (118). The discussion and mathematical derivations were based on recognizing the series of consecutive and competitive processes that would logically be involved after the administration of drug by several routes and on the assumption that the transfers effected would be by free or passive diffusion, i. e., diffusion according to Fick's law. With transfer by passive diffusion process, rates would be at all times directly proportional to the concentration of material present at a given transfer site, i. e., first-order processes.

Teorell derived expressions to calculate the amount-time course of drug at the absorption site, in the blood, in the tissues, amount eliminated, and amount inactivated. Since the treatment was general and included many processes, most of the mathematical expressions are rather complex, although less complex expressions based on simplifying assumptions are also presented.

Teorell also considered the special case of drop or continuous intravenous administration and derived the appropriate expressions to describe amount-time courses of drug in blood and tissues, as well as inactivated and excreted amounts under this condition of administration.

It is of interest to compare the theoretical treatment of Teorell to that of Dominguez. The kinetic situation considered by Teorell (3) may be depicted by the following scheme:



The k's with number subscripts are first-order rate or, as he described them, velocity constants. These constants are permeability coefficients divided by volume and, hence, have the dimensions of reciprocal time. For drug inactivation in the tissues, the rate constant has the same dimension even though the process is not necessarily rate limited by permeation. In Dominguez's work, for distribution, metabolism, and excretion after drug was in blood or plasma, the scheme that adequately described these processes was as given in the model immediately preceding Eq. 14. This description of the part of the overall drug disposition is probably the more correct of the two for it would usually be the case that drug inactivation would not take place in all of the other fluids of distribution as implied by Teorell's model, but in some particular tissue whose contribution toward the drug's total volume of distribution would be small. Adding another site in which destruction takes place to either model would be an even more correct description.

A large number of kinetic studies on absorption and excretion of drugs have been reported by Lapp in France. The bulk of the studies have been concerned with excretion kinetics and have included work on, or consideration of, several poisons (27); salicylate (28); stovarsol (29); uric acid (30); arsenic (31), sulfur, arsenic, chlorine, and sodium (32); rubiazol C (33); quinine (34); quinine and radioactive substances (35); arsenic mobilization in guinea pigs (36); a soluble bismuth compound (37, 38); sulfisoxazole (39); and N^1-acetylsulfisoxazole (40). Lapp has also published a number of papers concerned with other general or specific aspects of the subject of this review (41–51). For the most part, the kinetics of substances studied by Lapp were describable by the kinetics of Dominguez (10–15, 19–24) and/or Teorell (3). Since, in almost all cases, the materials studied by Lapp were drugs as such, rather than physio-

logical materials or materials similar chemically to physiological materials as studied by Dominguez, an important contribution of his work is to show that, in so far as their distribution and excretion in the intact body is concerned, the same principles apply for both classes of compounds. Exception to this statement exists, of course, in cases where specialized transport processes exist for physiological materials. But, even here, drugs exist that are handled by processes kinetically analogous to the physiological processes.

In addition to the usual kinetic parameters such as rate constants and/or half lives, Lapp defines an additional parameter which he calls "protorrhea" [rapidity of initial elimination (49, 50)]. Protorrhea, π, was defined as

$$\pi = 2.3\ Q_0\lambda \qquad \text{(Eq. 17)}$$

where Q_0 is amount of a dose eventually recovered in the urine and λ the rate constant for elimination from the body. From Eq. 10, λ is directly proportional to excretion rate at zero time if the dose instantaneously enters circulation and drug in blood is at equilibrium with drug in other fluids of distribution from zero time. Lapp's exponential expressions are based on powers of 10 rather than e, the base of the natural logarithms, hence his rate constants must be multiplied by 2.303 to convert them to the method of expression used here and most of the published literature. Since his half life, H, ("temps d'hémikrèse") is defined as $H = 0.30/\lambda$ where λ is the rate constant as calculated by him, half lives are identical to those defined here. Lapp points out the application of kinetic data in therapeutics and uses, in addition to the parameters already mentioned, another quantity called "transit" which is the quantity of a drug that is present in the body at a given moment.

Lapp's methods and terminology are used to some extent in France (52, 53).

A number of less extensive studies than those already discussed, but nevertheless contributory and informative contributions to the subject of *in vivo* kinetics, have been published.

Boxer and his co-workers have considered the kinetics of the rise and fall of drug blood level with repeated dosage at fixed intervals which is the usual dosage scheme in therapeutics (54). Assuming elimination of drug by a rate directly proportional to amount present, i. e., the usual case, and that the drug was given intravenously, they deduced that the plasma concentration immediately after the nth dose, Cp^n, would be given by

$$Cp^n = Cp^0(1 + r + r^2 + \ldots + r^{n-1}) \qquad \text{(Eq. 18)}$$

where $r = \exp[-K\Delta t]$, Cp^0 the maximum plasma level from a single dose, and Δt the dosage interval. Equation 18 is a geometric series and has the limit

$$Cp^\infty = \frac{Cp^0}{1 - r} \qquad \text{(Eq. 19)}$$

when r is less than 1 and n approaches infinity. Actually in most cases of drug administration, the limit is reached rather rapidly so the maximum concentration is also reached rapidly. Boxer, *et al.* (54), applied the expression to streptomycin administered to dogs and obtained good agreement between calculated and experimentally observed levels. Experimental applications of Eq. 19 or an equivalent have been made by Lapp (50), Butler and co-workers (58), and Swintosky, *et al.* (59). In two of these cases (58, 59), application was to oral dosage of phenobarbital and sulfaethylthiadiazole, respectively, and in the case of sulfaethylthiadiazole a modification was made in Eq. 19 to compensate for the delay in absorption that occurs when this route is used. Agreement between calculated and experimentally observed levels was good in both cases. Since the equilibrium level on either continuous dosage or dosage at fixed-time intervals can be expected to be reached rapidly, particularly if an initial "loading" dose is given, a method has been proposed to calculate dosage for sustained release products based on equating input and output rate when desired level of drug was in circulation (60).

With small values of K, the series that is Eq. 18 approaches its limit slowly. However, body level of drug after n doses may still be easily calculated, since the sum of a geometric series is given by

$$Cp^n = Cp^0\left(\frac{r^n - 1}{r - 1}\right) \qquad \text{(Eq. 20)}$$

where Cp^n is the level after n doses.

Although Eqs. 19 and 20 are written in terms of plasma concentration, they are equally correct if Ab, amount in the body, is substituted for concentration. If they are applied to estimate body level after oral dosage or dosage by some other route other than intravenous administration, the calculation will give the theoretical maximum amount that could be expected to be present. The actual amount would be less, depending on absorption and elimination rates. Nevertheless, because the theoretical maximum can be determined, the calculation provides useful information.

Somewhat similar to the situation existing when dosage is in fixed amounts and at fixed intervals of time is the case where drug is infused at

a constant rate as is sometimes used in therapeutics, kidney clearance determinations, and in experimental work. A quantitative consideration of this method of administration has been presented by Rand and Stafford (61). Their consideration was brought about by their difficulty in determining the acute lethal dose of rapidly detoxified drugs because this dose was a function of infusion rate. They derived an expression to determine the amount in the body of the experimental animal at death that was based on assuming a detoxification rate that was directly proportional to the amount present in the body and constant rate infusion. This expression was equivalent to

$$Ab^d = \frac{Ri}{K}(1 - \exp[-Kt]) \quad \text{(Eq. 21)}$$

where Ab^d was the amount in the body at death (true lethal dose), Ri the infusion rate, and K the detoxification rate. In the application of Eq. 21 to determine the lethal dose of digoxigenin in guinea pigs, they found the mean true lethal dose in the group of animals smaller and with about one-tenth the variance than the mean apparent lethal dose. The latter was calculated as just the product of infusion rate and length of time infusion proceeded before death.

Equation 21 expresses the dependence of amount of drug in the body on time when no drug is present at zero time. If an initial dose Ab^0 is introduced at zero time, the following expression gives the relation between amount in the body and time

$$Ab = \frac{Ri}{K}(1 - \exp[-Kt]) + Ab^0 \exp[-Kt] \quad \text{(Eq. 22)}$$

where the terms have the same meanings as in previous discussions. From Eq. 22 when $Ri = KAb^0$, $Ab = Ab^0$ for all values of time.

An application of the kinetic consideration contained in Eq. 22 and the discussion which follows it has been made by Mayer, Maickel, and Brodie to study the kinetics of the penetration of drug into the cerebrospinal fluid and brain (62). Since plasma level was constant at zero time, they were able to follow the appearance of drug in the other fluid of interest as a function of time. They found that penetration was by a passive process and, hence, were able to determine a relative permeability constant for the penetration.

Plasma corticoid levels following adrenocorticotropic hormone infusion have been analyzed by means of an analog computer programed for the equivalent of the differential form of Eq. 21 (63). The constant input rate Ri was the production rate of corticoids by the adrenal cortex and the removal rate was expressed by the equivalent of K.

Kinetic studies involving the fitting of experimental data to the equivalent of Eq. 14 or to more complex relationships have been reported. Paykoc and Powell have been able to describe the disappearance of trichloroacetate from the blood of humans by Eq. 14 with constants evaluated (64).

Portwich and Buttner (65) have fitted experimentally obtained serum sulfonamide level data obtained after intravenous administration of drug to the equivalent of Eq. 14. A different two-term exponential expression was applicable when drug was given orally. The latter equation was

$$Cp = Cp^0 \frac{k_1}{k_1 - k_2}(\exp[-k_2 t] - \exp[-k_1 t]) \quad \text{(Eq. 23)}$$

where k_2 was the elimination rate and k_1 the absorption rate. This equation assumes instantaneous attainment of equilibrium between drug in blood and drug in other fluids of distribution. A reasonable agreement between the theoretical curve and experimentally observed serum levels was obtained. However, it should be pointed out that Eq. 23 assumes that the absorption process was first order and this is not likely to have been the case, since slightly soluble sulfonamides are apparently rate-limited in absorption by the solution process at the absorption site (66). Solution rate-limited absorption would mean that absorption proceeded by a process based on the solution rate law appropriate for the case where surface area decreased as solution and absorption proceeded (67). It is of interest to point out that Eq. 23 was used in the same sense as used here, i. e., to describe body level when absorption and elimination rate were both directly dependent on concentration, by Teorell (3). He showed that this equation has a maximum corresponding to the time at which maximum body level occurred, and also showed the dependency of the value of the maximum level on the rate constants and dose. The latter relationship showed that maximum serum level was always directly proportional to the size of the dose.

Kalow (68) treated excretion rate data obtained after both the intravenous and subcutaneous administration of tubocurarine in terms of a kinetic model proposed by Teorell (3) to deal with the case (intravenous administration) wherein drug in blood passes to the tissues or other fluids of distribution in reversible exchange, drug in

blood is eliminated by the kidney, and drug in tissues undergoes destruction. When drug was given subcutaneously an additional process representing absorption was involved. Rather good agreement was obtained between experimental and predicted excretion. When drug was given intramuscularly as a suspension in oil and wax, it was not possible to describe the excretion rate curve by the mathematical expression which accounted for passive absorption from the injection site. Kalow (68) was able to explain previously reported experimental and clinical observations using tubocurarine from the results of the kinetic study.

The rate of disappearance of thiopental from plasma has been studied by Plough and his coworkers (69). Experimental data were empirically fitted to a three-term exponential expression. While the fit was relatively good, it is difficult to relate equation constants (coefficients of the exponential terms and of time) to physiological distribution or inactivation processes, since a number of models could be postulated which would, on integration of the differential expressions describing them, also consist of three exponential terms (*cf.* Eq. 14 and description of the values of various constants).

Several investigations of elementary kinetic considerations in the absorption and elimination of sulfaethylthiadiazole have been reported by Swintosky and his co-workers (59, 70–73). The findings in the kinetic studies were used in the design (72) and evaluation (73) of a sustained release dosage form of the drug. A study was also made on the sulfonamide sulfamethylthiadiazole (74).

The relationship expressed by Eq. 7 has been used to interpret the absorption of several drugs (75–77) and used as the basis for the development of a method (78) for calculating absorption rate from excretion rate measurements. This method was based in part on the determination of the first and second derivatives of the cumulative excretion curve.

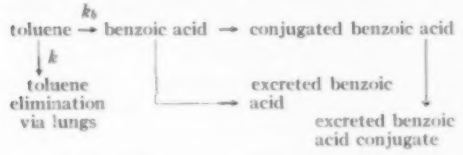
The kinetics of plasma drug level variation with time after oral administration of a controlled-release dosage form of drug and of drug in solution has recently been considered by Wiegand and Taylor (116). An expression was derived to account for first-order release of drug from dosage form followed by absorption, also by a first-order process with absorption followed by competitive first-order removal processes for urinary excretion and metabolism. The expression derived for solution dosage did not include a drug-release from dosage-form step. The more complex expression also accounted for the fact that only a portion of the dose contained in the special dosage form was released exponentially. Agreement between dog drug plasma levels experimentally observed and predicted by the expression derived for solution dosage was good. A less satisfactory fit of experimental plasma values to theoretically predicted values was obtained when the special dosage form was given. The rate constant used for the absorption step was eight times as large as the rate constant for release of drug from the dosage form, and it seems that if this large difference existed the slower step (release from dosage form) should have been the rate limiting one of the two processes. Neither expression accounted for stomach emptying as a process involved in absorption.

Not previously mentioned here, but of general interest in the field of *in vivo* kinetics, are studies on the distribution of insulin between two body water compartments (79); rate of exchange of substances between blood and extravascular fluid (80); theoretical consideration of transfer kinetics in multicompartment system (81); concentration time course of radio-mercury introduced as a mercurial diuretic (82); kinetics of radio-potassium in circulation (83); absorption rate of water from the stomach and small bowel of man (84); a short summary of values of, and discussion on, half life for drug elimination (103); kinetics of the transfer of blood expanders (85); absorption of *p*-aminohippuric acid (86); vitamin A disappearance from the plasma (87); interpretation of steroid half-life values (88); distribution kinetics of intravenous epinephrine (89); the behavior of intravenously injected particulate material (102); radioactive heparin in humans to establish a definite correlation between blood clotting time and radioactivity (90); studies on the disposition of digitoxin (121); the kinetics of the distribution of radioactive vitamin B_{12} (122); and a brief review on termination of drug action by elimination of unchanged drug (91). The kinetic basis for drug dosage schemes has been extensively discussed (100, 101).

A recent study on the kinetics of the disposition of the antibiotic, psicofuranine, made use of an analog computer for deducing rate constants from experimental data (120). The analog computer proved to be a powerful tool in the kinetic analysis. Rate constants were obtained for a complex model that included absorption from the stomach, stomach emptying, intestinal absorption, the reversible diffusion of drug into two body compartments, in addition to blood and urinary excretion.

KINETICS OF DRUG METABOLISM

Although Widmark (92) reported studies on the metabolism of alcohol nearly three decades ago, extensive studies on metabolism kinetics have been made only relatively recently by Bray and his colleagues at the University of Birmingham in England (93–99). The studies of Bray's group were extensive. Their first studies (94) were concerned with kinetics of the formation of benzoic acid from benzamide, toluene, benzyl alcohol, and benzaldehyde, and the conjugation of the acid formed with glycine and glucuronic acid. The experimental animal was the rabbit. Rate constants for the oxidation of precursors to benzoic acid were obtained by a mathematical analysis of the excretion curve which represented free and conjugated benzoic acid excretion. They assumed, and it proved to be the case, that formation of benzoic acid from precursor, conjugation of benzoic acid, and excretion of the benzoic acid conjugate all proceeded by first-order processes. The processes may be depicted as follows:



In order to determine the value of k_b, i. e., the rate constant for the formation of benzoic acid from toluene, the following procedure was used: It was assumed that conjugated benzoic acid was excreted much more rapidly than it was formed and, therefore, excretion was rate limited by the formation step. A plot of cumulative amount of benzoic acid and conjugates was constructed from the measured excretion of these substances. By determining, at several times after zero time, the difference between the ultimate maximum excretion of benzoic acid in all forms and excretion in the time chosen, they obtained values for the amount of toluene remaining in the body that was destined to be oxidized to benzoic acid. A semilogarithmic plot of these values *vs.* time gave a straight line. The rate constant, $K = k + k_b$, in the model shown above was obtained from the slope of this line (see Eq. 9 and the discussion preceding it). Since the proportion of the drug that was oxidized was known, it was easy to calculate k_b, the rate constant for oxidation, from the equivalent of Eq. 6. In the experiments where benzoic acid precursors were administered, glycine was also administered to insure that the formation of hippuric acid from benzoic acid would not be by a zero-order process.

The studies with benzoic acid precursors gave valuable information on the mechanism of toluene oxidation. There was evidence that benzyl alcohol was an intermediate in the oxidation of toluene. However, it was found that benzyl alcohol was oxidized to benzoic acid about three times more rapidly than benzaldehyde, the next logically expected intermediate. It was apparent then that this latter material could not have existed as such in the course of toluene or benzyl alcohol oxidation. It should be pointed out that if the oxidation of benzaldehyde is rate limited by diffusion to the site of oxidation this conclusion would be invalid. If benzaldehyde is an intermediate, it would be formed *in situ*.

Benzoic acid is conjugated with both glycine and glucuronic acid and experiments were conducted to determine the rates of these processes. Experimental animals were fed benzoic acid and the excretion of both metabolites followed. A plot of cumulative amount of hippuric acid excreted *vs.* time was linear, indicating that in the range of doses employed, hippuric acid was formed by a zero-order process. Formation of the ester glucuronide of benzoic acid was by a first-order process in competition with zero-order process resulting in the formation of hippuric acid. The value of the first-order rate constant, k_g, for the formation of the glucuronide was deduced in the following manner. A plot of benzoic acid excreted in all forms as a function of time was constructed. A plot of glucuronide excretion *vs.* time was also constructed. The slope of this curve was determined at several times and plotted against the difference between ultimate excretion of benzoic acid and excretion in the same time as determined from the first curve. The slope of the last curve was the first-order rate constant for formation of the glucuronide. The basis for the procedure follows from an examination of Eq. 24

$$dG/dt = k_g Ab \qquad \text{(Eq. 24)}$$

where dG/dt was the excretion rate of glucuronide and Ab was amount of benzoic acid in the body. When the subtraction involving the first curve was performed, Ab was obtained. The slope determination on the second curve gave dG/dt. Hence, k_g was the slope of the plot constructed as the third curve.

The body level at which first-order kinetics in the formation of hippuric acid would be observed was calculated. This level was dependent on the rate of glycine mobilization by the body.

It has been observed that substantially more benzoic acid conjugated with glucuronic acid was excreted when humans ingested sodium benzoate

as compared to the amount excreted when they ingested benzoic acid (104). The reason given was the more rapid absorption of benzoic acid from sodium benzoate (104). Benzoic acid can be expected to be absorbed more rapidly when given as the sodium salt than when given as the free acid (105). The kinetic studies of Bray's group allow an explanation for the phenomenon observed. It can be assumed that a limiting rate for mobilization of glycine exists in the human. With slow absorption following benzoic acid ingestion, the conjugation of benzoic acid with glycine could be expected to follow first-order kinetics and, since little glucuronic acid conjugate was excreted, this rate was substantially greater than the first-order rate of conjugation with glucuronic acid. With large amounts of benzoic acid in circulation as could be expected to have occurred when sodium benzoic acid was ingested, the conjugation with glycine could be expected to have proceeded by a zero-order process. High body level of benzoic acid would result in an increase in production of glucuronic acid conjugate because the rate of production is the product of the rate constant, as well as the body level. In order for an increase in ratio of glucuronic acid conjugate/glycine conjugate to be observed, it would only be necessary that the ratio $k_g/b/k_o$, where k_o is the zero-order rate constant for formation of hippurate, be greater than the ratio k_g/k_h, where k_h is the first-order rate constant for formation of hippurate for some period of time when zero-order kinetics described hippurate formation.

The kinetics of the formation of phenols from certain precursors were also studied by Bray and his co-workers (95). Since the rate limiting step in the sequence, precursors → phenol → conjugated phenol → excreted conjugated phenol was the hydroxylation to form the phenol, they were able to analyze metabolite excretion curves in a manner similar to that employed with benzoic acid precursors (94) to find the rate constant for this conversion. The materials studied included anisole, N-phenylurea, and benzoxazolone. The kinetics of the conjugation of phenols with glucuronic acid (96) and with sulfuric acid (97) was also studied. The results obtained indicated that the conjugation of phenols with glucuronic acid was by a first-order process. Materials studied included phenol, catechol, resorcinol, quinol, *m*- and *p*-methoxy-, *p*-chloro-, and *p*-cyanophenols, and 2,6- and 3,4-xylen-1-ols. The conjugation of phenols with sulfuric acid in the fasting rabbit was independent of the amount of phenol in the body above a certain body level, indicating that zero kinetics were followed (97). However, the simultaneous administration of sodium sulfite or L-cystine, which substances are converted to sulfate *in vivo*, changed the kinetics to first order.

A generalized mathematical treatment accounting for the metabolic fate of phenols, benzoic acid, and their precursors was presented later and accounted for the appearance of both zero-order and first-order kinetics in conjugation, depending on body level of the material being conjugated, in the course of a single experiment (98). Expressions were also presented to calculate the critical dose level of both the precursor and of the material formed, the latter being conjugated by two competitive processes, when zero-order kinetics could occur in one of the conjugation processes.

The conjugation of various *para*-substituted benzoic acid with glycine and glucuronic acid, the oxidation of *para*-substituted toluenes, and the hydrolysis of *para*-substituted benzamides were the subjects of kinetic studies in other work reported by Bray, *et al.* (99). With adequate supplies of glycine, where pertinent, all reactions were first order. No correlation was found between the magnitude of the velocity constants and the electronic nature or size of the substituent groups.

Kinetic studies on the metabolism of toluic acids and N-tolylureas were also reported (93). Due to slow absorption of tolylureas, conjugated metabolite excretion curves were linear for substantial periods of time during experiments. It was established that linearity of the curves resulted from the slow and constant rate absorption rather than a zero-order process in metabolism.

Some parts of the work of Bray and his group are reviewed in a book (117).

Butler and his co-workers have considered the kinetics of the *in vivo* demethylation of N-methylphenobarbital to phenobarbital (119). It appeared that appearance of phenobarbital in plasma could be considered as the formation of intermediate in a two-step, consecutive, first-order reaction. The first step of this process was demethylation and the second step metabolism and elimination of the phenobarbital formed.

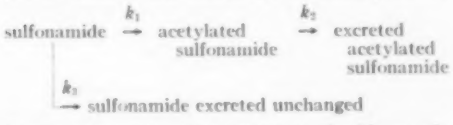
The kinetics of the excretion of the hippuric acid following administration of small doses of benzoic acid to humans has been studied by Elliott (106) and Elliott and Wu (107). It was found that hippuric acid excretion could be described as the last step of a three-step consecutive first-order process, the first step of which was benzoate absorption from the gastrointestinal tract, the second step conjugation with glycine, and the third representing excretion of hippuric acid. The value of the rate constant for hippuric acid formation was different in subjects whose pathology indicated liver disorders.

The kinetics of I^{131}-labeled diiodotyrosine metabolism has been studied, using normal human subjects (108). The kinetic model formulated accounted for plasma level of diiodotyrosine and rate of deiodination; saliva level, urinary excretion, and thyroid uptake of iodide-I^{131}.

The kinetics of ethanol elimination in man has recently been re-examined by Lundquist and Wolthers (109, 110). Widmark's early finding (92) was that alcohol disappears from the blood at constant rate after ingestion of moderate doses and their equilibration in body fluids. As reviewed by Lundquist and Wolthers (109), elimination of alcohol is by metabolism, urinary excretion, and by elimination by the lungs. The latter two processes involve only small amounts of material when moderate doses are administered. They also point out that at low levels, alcohol should not be removed at a constant rate and treated, the kinetics of removal to include such low levels in the terms of an enzyme-catalyzed process. Experimentally obtained alcohol level curves were fitted well by the appropriate theoretical expressions.

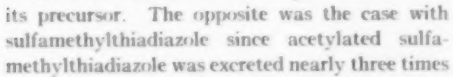
The rate constants for the conjugation of several amino and acetamido benzoic acids have recently been reported (111). Both dogs and rabbits were employed as experimental animals.

Studies on the kinetics of the excretion and acetylation of sulfisoxazole have been recently reported (112). The disposition of sulfisoxazole in humans was well described by considering that the process for excretion of free drug competed with the process for acetylation and that both processes were first order. Further, that excretion of acetylated drug was the second step of a two-step, consecutive, first-order process, the first step of which was acetylation. Appropriate mathematical expressions to fit this model were derived and applied to the experimentally obtained data. A similar model was found to describe the same processes for another sulfonamide, sulfamethylthiadiazole (113). The disposition scheme is shown diagrammatically below:



It was noteworthy that in the case of sulfisoxazole acetylation resulted in the formation of a substance more difficult for the body to remove than its precursor. The opposite was the case with sulfamethylthiadiazole since acetylated sulfamethylthiadiazole was excreted nearly three times more rapidly than sulfamethylthiadiazole itself.

The kinetics of the *in vivo* oxidation of tolbutamide to carboxytolbutamide and excretion of carboxytolbutamide were the subject of another recent study (114). The processes involved in the oxidation and excretion were consecutive first order. No competitive process was found for the oxidation step, nor for the urinary excretion of metabolite, since doses of either the precursor or metabolite were recovered nearly quantitatively in the urine as metabolite. Another study compared the oxidation rates of deuterated tolbutamide (N-*p*-benzotrideuteridesulfonyl N′-*n*-butylurea) to nondeuterated material. The *in vivo* oxidation rate was the same in both cases. The sequence of first-order processes observed in both cases was as follows:



In normal adult humans the ratio of rate constants, k/K, was about 12.

SUMMARY

The amount or concentration *vs.* time course of drugs in the body of the intact animal may many times be described by mathematical relationships derived from first principles concerned with the transport of material by diffusion and reaction kinetics even though many simplifying assumptions are implicit in the derivations. The usefulness of quantitative information of this nature in therapeutics is obvious but less obvious is the potential of such information to help understand the mechanisms by which drugs act, the mechanisms by which the body modifies and eliminates drugs, and the relationship between drug concentration and activity.

Detailed kinetic studies have usually not been made in the course of early investigations on new drugs but there are indications that they will be made more frequently in the future. These studies should, at the very least, eliminate much routine testing. For example, benzylpenicillin in use now for nearly two decades has never been the subject of a detailed kinetic study, yet thousands of tests have been made to determine serum levels under various conditions of administration. A detailed kinetic study on not more than 12 test subjects would provide more information on the absorption, distribution, and elimination of this drug than all the studies made to now.

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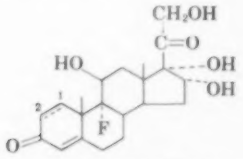
Steroid Borates I. Structural Considerations

By LEWIS J. LEESON, JAMES A. LOWERY, GEORGE M. SIEGER, and SIEGFRIED MULLER

It has been found that alkaline aqueous solutions of various boric acids readily solubilize steroid molecules containing a *cis*-1,2-diol grouping. Both triamcinolone (9α-fluoro-16α-hydroxy-prednisolone) and its synthetic precursor "F-3" (9α-fluoro-16α-hydroxy-hydrocortisone) demonstrate this property. It is proposed that the structure of the product formed between the steroid and alkaline borate involves two steroid molecules and one tetrahedral boron atom. Some physicochemical data are presented to substantiate this view.

THE RELATIVELY low aqueous solubility of adrenocortical steroid hormones has often been a hindrance in preparing certain pharmaceutical formulations. Chemical modifications for increased water solubility have been mostly confined to variations at the 21 carbon atom. Such derivatives as the 21-hemisuccinate, hemiphthalate, and hemimaleate (1) as well as the phosphate (2) have been prepared and converted to their water-soluble sodium salts. In addition, 21-pyridinium and diethylaminoacetate hydrochloride (3, 4) as well as sodium salicylate complexes (5) have been used to enhance aqueous solubility of steroids. In attempting to find water-soluble forms of triamcinolone[1] suitable for pharmaceutical use, the steroid borates were prepared and characterized. In this report triamcinolone (Ib) will often be referred to as F-4 and its synthetic precursor, 1,2-dihydro-triamcinolone (Ia) will be designated as F-3.



Ia—Carbons 1,2: CH_2—CH_2 (F-3)
Ib—Carbons 1,2: CH=CH (triamcinolone: F-4)

EXPERIMENTAL

Preparation of F-3 and F-4 Borate Salts with Alkaline Tetraborates.—Six grams of F-3 was added to 50 ml. of 0.12 *N* aqueous $Na_2B_4O_7$ solution. After mixing overnight, the undissolved material was removed by filtration, and the filtrate lyophilized. The white solid obtained was recrystallized from acetone.

Both F-3 and F-4 sodium and ammonium borates were prepared in this manner. F-4 sodium borate could not be recrystallized. Elemental analyses, to be discussed later, are found in Table I.

Preparation of F-3 Cycloborate and Its Corresponding Sodium Salt by the Thomas Procedure (6).—A solution of 600 mg. of F-3, 3 Gm. of boric oxide, and 15 ml. methanol were refluxed for one hour. The mixture was diluted with 90 ml. of distilled water and permitted to stand overnight at room temperature, when 541 milligrams of needle-like crystals were obtained and recrystallized from acetone-*n*-hexane.

Sodium hydroxide solution (4 ml. of 0.1 *N*) was added to a suspension of these crystals in methanol. After shaking for one-half hour, the undissolved material was removed by filtration and the product was isolated from the filtrate by lyophilization.

Attempts to prepare F-4 borate by this procedure were unsuccessful.

Determination of Neutralization Equivalent of F-3 Cycloborate.—Potentiometric titrations of various samples of F-3 cycloborate were performed by suspending each sample in approximately 20 ml. of water and titrating with standard alkali solution. In all cases it was observed that complete solubilization of the steroid coincided with the potentiometric end point.

In all experiments mentioned in this report the titrations and pH values were performed with a Beckman model G pH meter equipped with calomel and glass electrodes. All end points were evaluated by the method of double derivatives (7).

Determination of Molar Ratio of Steroid to Sodium Tetraborate by Titrimetric Means.—Three samples each of F-3 and F-4 were suspended in 20 to 25 ml. of water and titrated with 0.1213 *N* aqueous $Na_2B_4O_7$ solution. Because of the rate at which the steroid is solubilized, it was necessary to wait some time until equilibrium was established (fifteen to thirty minutes) before the next volume of borate solution was added. In all cases, clearing of the solutions was observed near the potentiometric end point. From these values the molar ratio of steroid to sodium borate was determined.

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[1] Lederle Laboratories trade name for triamcinolone is Aristocort.

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Table I—F-3 Borate Salts

Description of Steroid Borate	Empirical Formula, *Anal.*—Calcd. for	C	H	F	B	Na	N
Steroid:boron molar ratio 1:1 as sodium salt (IIa)	$C_{21}H_{28}BFNaO_7$	56.77	6.13	4.28	2.44	5.18	...
Steroid:boron molar ratio 2:1 as sodium salt (IIIa)	$C_{42}H_{56}BNaF_2O_{12}$	61.31	6.62	4.62	1.32	2.79	...
Found on preparation A		60.94	7.04	4.66	1.24	3.24	...
Found on preparation B		60.30	7.02	4.66	0.92	2.93	...
Steroid:boron molar ratio 1:1 as ammonium salt (IIa)	$C_{21}H_{29}BFNO_7$	57.41	7.11	4.33	2.46	...	3.19
Steroid:boron molar ratio 2:1 as ammonium salt (IIIa)	$C_{42}H_{58}BF_2NO_{12}$	61.68	7.15	4.65	1.32	...	1.71
Found on preparation C		61.42	8.10	...	0.91	...	1.58

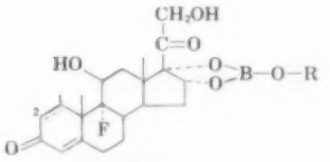
Determination of Molar Ratio of Steroid to Sodium Tetraborate by U. V. Analysis.—To each of ten flasks was added exactly 10.0 ml. of aqueous 0.1213 *N* sodium tetraborate solution. These flasks were divided into two groups of five and to each flask within a group was added varying amounts of water. This resulted in solutions containing the same absolute amount but different concentrations of sodium tetraborate. The volumes of water added were 0.0, 10.0, 25.0, 40.0, and 75.0 ml. To each of the first group of five flasks was added 1.1 Gm. of F-3 while to the second group 1.1 Gm. of F-4 was introduced. The mixtures of steroid and sodium tetraborate solutions were permitted to stand for two to three days with occasional shaking. At the end of this time the excess steroid was removed by filtration and, after proper dilution, the amount of solubilized steroid was determined on a Beckman DU spectrophotometer at 240 mμ, using a reagent blank.

Determination of Molar Ratio of Steroid to Boric Acid by the Böeseken Technique (8).—Four F-4 samples of varying weights were each added to a 5-ml. volumetric flask. Two milliliters of a saturated solution of boric acid in methanol was introduced and the flasks filled to the desired volume with methanol. One milliliter of each solution was added to a 100-ml. volumetric flask, filled to volume with distilled water, and the pH of the resulting solution determined. A similar experiment was performed with three F-3 samples.

DISCUSSION

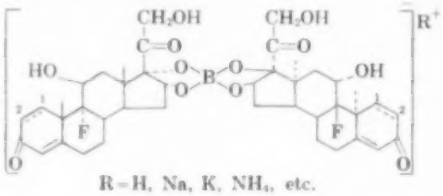
In early investigations with triamcinolone-like steroids, it was recognized that the 16α-17α-*cis*-diol grouping should combine with boric acid in the same way that boric acid complexes with glycerol (9) pentaerythritol (10, 11) and other polyols. Although it was soon discovered that boric acid solutions, *per se*, did not markedly enhance the aqueous solubility of F-3 and F-4, alkaline borates did. The soluble product was isolated by lyophilization.

During the course of our investigations a description of the synthesis of steroid cycloborates of general structure II was disclosed (6). Employing this procedure, both F-3 cycloborate and its sodium salt were prepared. Infrared spectra appeared to indicate that this was the same material prepared in these laboratories when F-3 was treated with aqueous sodium tetraborate solution.



R = H, Na, CH_3, etc.
IIa—Carbons 1,2: CH_2—CH_2
IIb—Carbons 1,2: CH=CH
1:1 Steroid to Boron Structure

In carrying out characterization studies on the steroid borates it was felt that using elemental analysis alone as a basis of structure was inadequate and unreliable. Accordingly, some physicochemical techniques were employed to determine the nature of these complexes. As a result of our investigations, general structure III has been proposed for the steroid borates.



R=H, Na, K, NH_4, etc.

IIIa—Carbons 1,2: CH_2—CH_2 (F-3 Borates)
IIIb—Carbons 1,2: CH=CH (F-4 Borates)
2:1 (bis) Steroid to Boron Structure

The subject of polyol-borate complexes is one in which much disagreement is encountered. Numerous investigators (12–19) have studied these materials and an examination of the literature indicates that their structures are not well defined. Therefore, since opinions on the nature of polyol-borate complexes are divided with respect to them being composed of either a 1:1 or 2:1 polyol to boron molar ratio, this report will discuss all experimental data with consideration to both of these structural possibilities.

Elemental Analysis and Neutralization Equivalent.—The elemental analyses for F-3 sodium and ammonium borates are listed in Table I. It can be seen that these results are not confirmatory for either of the two structures proposed, although they tend to favor the 2:1 configuration. Although

elemental analysis is usually an important tool in proof of structure, with steroid borates these data were considered to be of limited value. The reasons for this conclusion are summarized as follows: (*a*) The bonds between steroid and boron are not covalent in nature, but appear to involve weaker forces. Thus, analytically pure samples are difficult to obtain. (*b*) In general the presence of fluorine in a molecule interferes with the analysis for boron (20, 21). (*c*) The methods of preparing F-3 borate (free acid) result in a product contaminated with boric acid. This material cannot be purified readily.

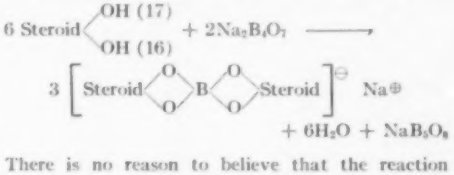
However, in spite of the above limitations, some useful observations may be made from elemental analyses listed in Table I, if these data are examined in light of the two possible structures. The results for sodium and nitrogen agree fairly well with the theoretical values for the 2:1 complex whereas they are only half the amount necessary to satisfy the 1:1 structure. However, this material is completely water soluble, indicative of salt formation. Furthermore, calculating the ratio of per cent carbon to sodium or nitrogen yields results which more markedly demonstrate the greater possibility of a 2:1 structure. These values are listed in Table II.

The neutralization equivalent data listed in Table III also favor a 2:1 structure since they more closely approximate the value of 801 rather than that of 410 for the 1:1 complex. However, these results would be expected to be somewhat low since, as mentioned previously, this material was contaminated with boric acid. The procedure for determining these values, described in the experimental section, was such that any boric acid present as a contaminant would be titrated separately with an end point at approximately pH 10, since it was not complexed with a *cis*-diol (22). Figure 1, a typical titration curve obtained in these experiments, shows the presence of this possible second end point. Furthermore, the initial potentiometric end point coincided with complete solubilization of the steroid.

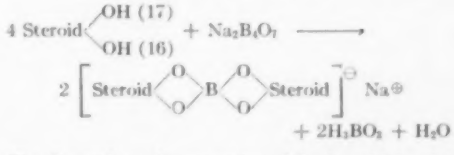
The results of the titrimetric neutral equivalent experiments appear to be supported by preliminary molecular weight determinations[2] employing a vapor pressure-thermistor method in combination with conductivity measurements.

Molar Reaction Ratio of Steroid to Sodium Tetraborate via Titrimetric and Ultraviolet Analyses. —One of the strongest arguments against the 1:1 structure is to be found in the experiments in which suspension of F-3 and F-4 were titrated with sodium tetraborate. From the potentiometric end points, which corresponded well with the observed steroid solubilization, the molar reaction ratios of steroid to sodium tetraborate were determined. In Table IV it can be seen that the molar combining ratio of either F-3 or F-4 to sodium tetraborate was found to be 3:1. These results raise serious doubts concerning a structure based on a 1:1 ratio of steroid to boron (IIa and b), for although there are a sufficient number of boron atoms present for such a structure, there are only two sodium atoms available to solubilize three molecules of steroid.

A molar reaction ratio of three to one, however, although not confirmatory, does not negate the structure proposed by the authors (IIIa and b), since the reaction between steroid and sodium tetraborate could also be projected as follows:



There is no reason to believe that the reaction between steroid and sodium tetraborate necessarily involves a complete combination of the two materials, and the formation of sodium pentaborate as a reaction product might be used to balance the equation. If this were the case, it could be asked why the pentaborate did not react with another two moles of steroid resulting in an overall reaction involving a molar combining ratio of 4:1 as shown:



The formation of two moles of boric acid in the above reaction is to be expected since it was demonstrated by Kahlenberg and Schreiner that aqueous sodium tetraborate solution is equivalent to equimolar amounts of sodium borate and boric acid (23).

TABLE II.—RATIO OF PER CENT CARBON TO SODIUM AND NITROGEN

Ratio of Steroid to Boron	% C/%Na Theory	% C/%Na Found	% C/% N Theory	% C/% N Found
1:1	10.9		18.0	
		18.8		
		20.6		39.0
2:1	22.0		36.0	

TABLE III.—TITRATION OF F-3 CYCLOBORATE WITH DILUTE AQUEOUS SODIUM HYDROXIDE

Sample Weight, mg.	Base Employed, *N*	End Point, ml.	Neutral Equivalent
344.3	0.0815	5.441	776
446.2	0.1186	4.788	786
275.0	0.0819	4.210	745

TABLE IV.—MOLAR RATIO OF STEROID TO SODIUM TETRABORATE BY TITRATION

Steroid	Moles Steroid $\times 10^{-4}$	Moles $Na_2B_4O_7$ Used $\times 10^{-4}$	Molar Ratio of Steroid: $Na_2B_4O_7$
F-4	1.25	4.14	3.0/1
	2.41	7.99	3.0/1
	1.35	4.74	3.0/1
F-3	1.80	6.23	2.9/1
	2.48	8.42	2.9/1
	2.68	9.18	2.9/1

[2] This method was presented by A. Wilson, *et al.*, at the American Chemical Society Meeting-in-Miniature, Seton Hall, East Orange, N. J., January 1959.

The answer to why a 4:1 reacting ratio was not observed could lie in the fact that the pentaborate is not nearly as strong an alkaline salt as the tetraborate (24). As a result, the concentration of pentaborate may have been two low for it to be effective in the comparatively short time required for the titration.

To test the possibility of a 4:1 combining ratio, the determination of the molar ratio of steroid to sodium tetraborate by ultraviolet analysis was performed. In this experiment, although the same total amount of sodium tetraborate was employed to solubilize various F-3 and F-4 samples, the borate was present in the solution in varying concentrations. The results, seen in Table V, indicate that in the most concentrated tetraborate solutions the molar reaction ratios of steroid to sodium tetraborate are four to one. In general, as the borate concentrations decrease, the ratios vary from 4:1 to values between 3:1 and 4:1. These fractional values in the dilute solutions might be attributed to some solubilization by the pentaborate formed, since in these experiments the suspensions were permitted to equilibrate for from two to three days. This is a longer period than the time required for the previously described titrations. The possibility of this solubility being a result of decomposition has been negated by stability studies of these complexes. Over a pH range of 7.5–10 steroid borates are stable for the time periods mentioned above.

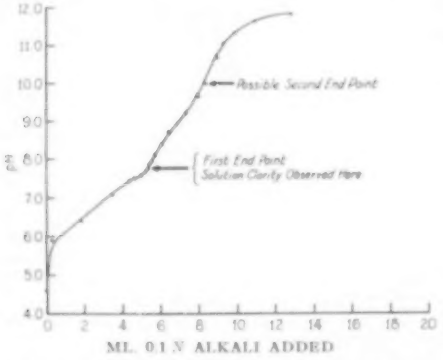


Fig. 1.—Titration of the F-3 borate suspension.

Table V.—Determination of Steroid to Sodium Tetraborate by U. V. Analysis

Steroid	Volume of Solution Containing 1.213 meq. of $Na_2B_4O_7$	Steroid Solubilized, mg.	Molar Ratio of Steroid: $Na_2B_4O_7$
F-4	10	959.4	4.0/1
	20	954.4	4.0/1
	35	845.1	3.5/1
	50	906.4	3.8/1
	85	853.1	3.6/1
F-3	10	945.4	4.0/1
	20	850.4	3.5/1
	35	805.1	3.4/1
	50	805.1	3.4/1
	85	821.1	3.4/1

Ratio of Steroid to Boric Acid by the Böeseken Techniques.—In the literature numerous references can be found concerning the molar combining ratio of polyols and boric acid (12–16); however, the most complete and generally accepted work in this field is that of Böeseken, *et al.* (8, 17–19, 25). In his investigations, three different physicochemical techniques were employed for determination of the molar combining ratio of polyols and boric acid, with all methods yielding the same results. Of the three techniques, the procedure most readily adaptable to our problem involved the measurement of pH values of aqueous solutions of polyols and boric acid.

Böeseken based this technique on the difference in pH values between two solutions containing identical amounts of boric acid, but varying concentrations of *cis*-diol. Equation 1 was derived as a function of these conditions

$$-\Delta \text{ pH} = n/2 \log a \qquad \text{(Eq. 1)}$$

Δ pH, difference in pH between two solutions containing identical concentrations of boric acid and varying concentrations of *cis*-diol (steroid); a, ratio of *cis*-diol (steroid) concentrations in the two solutions; n, moles of *cis*-diol (steroid) per mole of boric acid.

Using this experimental technique, the pH values listed in Table VI were obtained with various solutions of steroid and boric acid. Employing Eq. 1, values of n were calculated. These results, found in Table VII, show that within experimental error the value of n for F-3 and F-4 is 2, in agreement with the *bis* steroid tetrahedral boron configuration.

Table VI.—Tabulation of Data for Equation 1

	Sample Number	pH	Weight of Steroid, Gm.	Concentration of Steroid, γ/ml.	Concentration of Boric Acid, γ/ml.	Molar Ratio of Steroid to Boric Acid
F-4	1	4.31	0.3370	674	747	0.141/1.0
	2	4.65	0.1721	344	747	0.072/1.0
	3	5.00	0.0698	140	747	0.029/1.0
	4	5.38	0.0326	66.4	747	0.014/1.0
F-3	1	5.71	0.0719	71.9	747	0.150/1.0
	2	5.36	0.1630	163	747	0.0340/1.0
	3	4.80	0.5896	590	747	0.123/1.0

TABLE VII.—TABULATION OF DATA OBTAINED USING EQUATION 1

	Sample Combinations	Ratio of Steroid Concentrations a	$-\Delta$ pH	n	Average n ($\bar{n}$) and Standard Deviation (s)
F-4	1 and 2	1.96	0.34	2.32	$\bar{n} = 2.06$
	1 and 3	4.83	0.69	2.02	$s = 0.2$
	1 and 4	10.15	1.07	1.82	
	2 and 3	2.47	0.35	1.79	
	2 and 4	5.18	0.73	2.04	
	3 and 4	2.10	0.38	2.36	
F-3	1 and 2	2.23	0.35	1.96	$n = 1.99$
	1 and 3	3.62	0.56	2.00	$s = 0.02$
	2 and 3	8.20	0.91	2.00	

SUMMARY AND CONCLUSIONS

1. The preparation of water-soluble sodium and/or ammonium borate derivatives of 9α-fluoro-16α-hydroxy prednisolone (triamcinolone) and 9α-fluoro-16α-hydroxy hydrocortisone has been described.

2. A number of analytical and characterization studies on these borates have been carried out in an attempt to learn more about their chemical nature and to prove their structure. Of two possible alternative structures, II and III, the latter appears to satisfy most adequately all of the experimental data.

3. Experimental techniques used as a basis for selection of the most acceptable steroid borate structure include: (*a*) Microanalytical elemental assays emphasizing carbon to sodium and carbon to nitrogen ratios. (*b*) Molecular weight determinations by a titrimetric neutralization equivalent method. (*c*) Determination of combining molar ratio of steroid to boric acid by the Böeseken technique. (*d*) Evaluation of the stoichiometric combining quantities of steroid and sodium tetraborate by titrimetric and ultraviolet analyses.

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Study of the Polyvinyl Alcohol-Borate-Iodine Complex II. Test Papers

By ANTHONY J. MONTE-BOVI and JOHN J. SCIARRA

Boric acid and acidified solutions of borates have been qualitatively detected by the time-honored test utilizing "turmeric paper." While adequate, the test does present some difficulty in its interpretation. Unless certain precautions are taken the results of this test may be misleading. Recently, it was shown that solutions of polyvinyl alcohol and iodine would produce a blue color when added to solutions of borates. Strips of filter paper impregnated with a solution of polyvinyl alcohol and iodine, and allowed to dry, also give a blue color when a drop of acidified borate is added. However since starch will also give a blue color in the presence of iodine, samples of borate containing starch (borate dusting powders, powdered boric acid) must be first treated with pancreatin or other enzymes which will render the starch inactive against iodine. The same principles can be extended to the use of a paper impregnated with a solution of polyvinyl alcohol and boric acid for the detection of iodine; while paper impregnated with boric acid and iodine can be used as a qualitative test for polyvinyl alcohol. Starch must also be rendered inactive when present with the polyvinyl alcohol. These test papers provide a rapid, reliable, and accurate means for the detection of borates, iodine, or polyvinyl alcohol.

TURMERIC PAPER has been used for many years for the qualitative detection of boric acid and other borates. While adequate, this test does present some difficulties and unless interpreted correctly, the results may be misleading. When utilized according to the method suggested by the official compendia (1, 2), a drop of 5 per cent boric acid (pH about 5 at ambient room temperature) when touched to a strip of turmeric paper, darkens the paper but not any more than a drop of distilled water (pH about 5 at ambient room temperature). The darkening of the turmeric paper is, in effect, due to the wetting action of the solution and not due to the borate present. At this point it is difficult, if not practically impossible, to distinguish between the turmeric paper moistened with the boric acid solution and the paper moistened with the distilled water. Upon drying, however, the turmeric paper with the boric acid added does show a very faint, pale pink or flesh colored discoloration. The official compendia describes this color as being "brownish-red," which may be misleading since the color seems to be more of a pastel shade of a peach or flesh-pink hue. The turmeric paper wetted with water dries to its original yellow color, without any sign of discoloration. Repeated tests have indicated that it is better to interpret the test after the turmeric paper has been dried. Examination of the spot produced under magnification, with an ordinary hand magnifier or reading glass, aids in detecting the flesh-pink discoloration.

The official directions for this test specify that a drop of ammonia T. S. be added to the spot produced on the turmeric paper by the addition of boric acid. A pronounced change in color occurs although the color produced is very similar to the color produced when a drop of ammonia T. S. is added to turmeric paper without the boric acid spot. Instead of the expected "greenish-black" color suggested by the official test, the color seems to be more of a faint or pastel shade of olive-green. It is difficult, at this point, to differentiate between the spot produced by the ammonia T. S. on the boric acid-turmeric paper, and that produced by the ammonia T. S. on the turmeric paper wet with distilled water. Upon drying, the olive-green color is gradually lost and the color of the spot before the addition of the ammonia T. S. is restored, e. g., flesh-pink with the boric acid-turmeric paper, and yellow with the turmeric paper alone.

Borates (sodium borate and sodium perborate), when acidified with hydrochloric acid to a pH of about 5 at ambient room temperature, give essentially the same results. While this test is in common use, the results obtained are questionable.

Recently, it was noted that polyvinyl alcohol and iodine solutions produced colors ranging from wine-red to deep blue-black when reacted with acidified borates (3), the color produced depending upon the concentration and grade of polyvinyl alcohol used. This reaction was used as a basis for the qualitative detection of boric acid and borates (4).

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In light of the above developments, it was decided to prepare test papers impregnated with solutions of polyvinyl alcohol and iodine and to test the performance of this paper as a rapid, accurate, and reliable method for the detection of borates.

EXPERIMENTAL

Preparation of the Test Paper.—Sheets of porous filter paper which in itself did not produce a blue color with iodine (e. g., Whatman No. 3 MM and Whatman No. 40 or their equivalents) were utilized as the base for the polyvinyl alcohol-iodine solution. The paper was then streaked or dipped into a solution containing equal parts of 0.1% iodine solution and 2% polyvinyl alcohol, allowed to air dry, and then cut into small strips of convenient size. When kept in a tightly stoppered bottle, the test papers retained their characteristics for at least one year at room temperature. To date no evidence of decomposition has been noted. The color of the dried filter paper strips varied from the original white color to a light to medium reddish-brown color depending upon the concentration and grade of polyvinyl alcohol used. The colors produced are given in Table I.

TABLE I.—COLOR OF PVA – Iodine – BORATE COMPLEX

Polyvinyl Alcohol—Grade	Color of Filter Paper			
	(PVA + I_2)		PVA + I_2 + Boric Acid	
Elvanol[a]	Wet	Dry	Wet	Dry
50-42	Red-brown	Lt. brown	Lt. blue	Color faded
51-05	Red-brown	Lt. brown	Lt. blue	Color faded
52-22	Red-brown	Lt. brown	Lt. blue	Color faded
70-05	Yellow	White	No change	No change
71-24	Yellow	White	No change	No change
71-30	Lt. green	White	No change	No change
72-51	Yellow	White	No change	No change
72-60	Yellow	White	No change	No change
Vinol[b]				
PA-5	Red-brown	Lt. brown	Lt. blue	Color faded
PA-20	Red-brown	Lt. brown	Lt. blue	Color faded
PA-40	Red-brown	Lt. brown	Lt. blue	Color faded
PA-100	Red-brown	Lt. brown	Lt. blue	Color faded
FH-100	Yellow	Colorless	No change	No change
FH-400	Yellow	Colorless	No change	No change
FH-500	Yellow	Colorless	No change	No change
FH-600	Yellow	Colorless	No change	No change
FH-602	Yellow	Colorless	No change	No change
FH-1500	Yellow	Colorless	No change	No change

[a] Elvanol, E. I. duPont de Nemours and Co., Inc., Wilmington 98, Del.
[b] Vinol, Colton Chemical Co., Cleveland 14, Ohio.

Preparation of Solutions.—The iodine solution was prepared by dissolving 1 Gm. of iodine U. S. P. in enough distilled water (containing 2 Gm. of potassium iodide U. S. P.) to make 1 L. of solution. The polyvinyl alcohol solutions were prepared by dissolving 2 Gm. of the appropriate polyvinyl alcohol in enough water to make 100 ml. of solution. Most of the solutions must be heated to a temperature between 80–95° to effect complete solution (5, 6). Others will dissolve at room temperature.

Just prior to impregnating the filter paper, these solutions were mixed in equal quantities and used to prepare the test papers.

Testing Procedure.—A drop of 5% boric acid solution was added to the polyvinyl alcohol-iodine test paper. The colors produced are noted in Table I.

The procedure was repeated using solutions of sodium borate and sodium perborate acidified with hydrochloric acid to pH of about 5 at ambient room temperature. Similar results were obtained.

In order to increase the intensity of the blue color produced the procedure was repeated using several grades of polyvinyl alcohol at a concentration of 10%. Elvanol 51-05 and Vinol PA-20 were selected as representative grades of partially acetylated polyvinyl alcohol. Other partially acetylated grades are also suitable for use. These results are shown in Table II.

TABLE II.—SENSITIVITY OF PVA[a] – I_2 TEST PAPER

Concentration of Boric Acid, % w/v	Color of Filter Paper			
	Elvanol 51-05		Vinol	PA-20
	Wet	Dry	Wet	Dry
0	Red brown	Red brown	Red brown	Red brown
1	Slight blue	Slight blue	Slight blue	Slight blue
2	Lt. blue	Lt. blue	Lt. blue	Lt. blue
3	Deep blue	Deep blue	Deep blue	Deep blue
4	Deep blue	Deep blue	Deep blue	Deep blue
5	Deep blue	Deep blue	Deep blue	Deep blue

[a] Ten per cent PVA.

Starch, if present, may give a blue-black color with the iodine in the test paper. With higher concentrations of polyvinyl alcohol the iodine is combined as a complex with the polyvinyl alcohol and will not give a blue color with starch. In the event that free iodine is present in the test paper then boric acid and borate samples containing starch must be treated. A drop of iodine T. S. was added to the boric acid or borate solution suspected of containing starch. If starch is present a blue-black color would form. This solution should be made slightly alkaline (pH about 8) with dilute sodium hydroxide T. S. followed by the addition of about 0.05–0.10 Gm. of pancreatin N. F. (one part pancreatin to 25 parts starch). This is then heated to 40° for five to ten minutes. The starch should be completely hydrolyzed to maltose by this treatment and will no longer give a blue-black color with iodine T. S. The resulting solution is reacidified to pH of 5 and applied to the polyvinyl alcohol-iodine test paper. In this procedure it is essential that an excess of pancreatin in amounts to hydrolyze the starch be maintained.

DISCUSSION

As can be noted from Table I only certain grades of polyvinyl alcohol produce a blue color with borates in the presence of iodine. It has been found that the partially hydrolyzed or partially acetylated grades of polyvinyl alcohol give the characteristic blue color with borates and iodine. Table III

Table III.—Per Cent Hydrolysis of Polyvinyl Alcohols

Grade	Viscosity, c.p.s.	Hydrolysis, %
Elvanol		
50-42	35–45	87–89
51-05	4–6	88–89
52-22	21–25	87–89
70-05	4–6	99–100
71-24	23–28	97.7–98.4
71-30	28–32	99–100
72-51	45–55	97.7–98.4
72-60	55–65	99–100
Vinol		
PA-5	4–6	86–89
PA-20	19–25	86–89
PA-40	35–45	86–89
PA-100	90–110	86–89
FH-100	4–6	98.5–100
FH-400	22–32	98.5–100
FH-500	34–50	98.0–98.6
FH-600	50–70	98.5–100
FH-602	48–70	98.0–98.6
FH-1500	140–155	98.5–100

shows the per cent hydrolysis of some of the common grades of polyvinyl alcohol commercially available. Viscosity is included in this Table only to point out the differences among the various grades in each group.

While a 2% polyvinyl alcohol solution gives somewhat satisfactory results it has been found that a 10% solution will increase the sensitivity of the test so that solutions containing at least 2% of borate present can be readily detected. A 1% concentration produces positive results although not as pronounced as the higher concentrations of borate.

As would be expected, paper impregnated with a mixture of equal parts of 5% boric acid and 2% polyvinyl alcohol (partially acetylated grade such as Elvanol 51-05 or Vinol PA-20) solution can be used to detect iodine in solution, since a drop of iodine solution added to this paper will produce a blue color, the intensity of which will depend upon the concentration of iodine in the solution being tested. In making this test paper it is better first to add the boric acid solution to the paper, allow it to dry, then add the polyvinyl alcohol solution, and again allow to dry. Boric acid will cause a precipitation of polyvinyl alcohol when in high concentration.

Paper impregnated with a mixture of equal parts of 5% boric acid and 0.1% iodine (1 Gm. iodine, 2 Gm. potassium iodide per L.) solution when allowed to dry can be used to detect polyvinyl alcohol in solution, since a drop of such solutions will produce a distinct color. In this case the color produced will vary not only with the concentration of the polyvinyl alcohol but also with the various commercially available grades which differ in the percentage of acetyl groups remaining in the polyvinyl alcohol polymer. In such cases it is advisable to run a series of control tests using the various grades of polyvinyl alcohol. Starch may also interfere and therefore must be rendered inactive by the use of pancreatin, as previously described.

SUMMARY

1. A test paper consisting of polyvinyl alcohol and iodine has been developed and found to be useful for the qualitative detection of boric acid and borates. This test paper has been found to be reliable, accurate, and rapid as compared to the use of turmeric paper test.

2. Additional test papers consisting of polyvinyl alcohol-boric acid and boric acid-iodine have been developed. These have been found useful for the detection of iodine and polyvinyl alcohol, respectively.

3. It has been found that the partially hydrolyzed or partially acetylated grades of polyvinyl alcohol produce a deep blue color with borates. Therefore, it is suggested that a grade of polyvinyl alcohol in this group be used for the detection of borates.

4. While a 2 per cent polyvinyl alcohol solution has been found to yield satisfactory results, it is suggested that a 10 per cent solution be used for this purpose.

5. Starch, if present as an interfering substance, can be rendered inactive by digestion with pancreatin or other starch digesting enzymes.

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Analysis of the Alkaloid Content of Ergot

By THOMAS G. ALEXANDER and DANIEL BANES

A new procedure for the assay of ergot is presented. Total alkaloids are extracted with ammonia-methanol-chloroform and water-soluble alkaloids are separated by column chromatography. Total and water-soluble ergot alkaloid contents are determined colorimetrically. The relative concentrations of ergonovine and ergonovinine in the water-soluble alkaloid fraction are estimated by paper chromatography. The new procedure yields higher values for water-soluble alkaloid content than does the current N. F. procedure.

THE NATIONAL FORMULARY method (1) for the determination of the alkaloid content of ergot has several drawbacks. Extraction of total alkaloids from the ergot is sometimes incomplete under the conditions prescribed, and emulsions are often formed during the extraction, making the procedure tedious and time-consuming. In a collaborative study on a modified version of the N. F. procedure, Strong and Maurina (2) found a large variation in results reported from different laboratories for the alkaloid content of ergot samples. This study was undertaken in an effort to reduce variability in assay, and to permit a more accurate evaluation of ergot samples with respect to their ergonovine content.

Various methods for extracting alkaloids from ergot were investigated (3, 4, 5). The method proposed here involves a simple extraction of total alkaloids by shaking ergot with two immiscible solvents. Water-soluble alkaloids are then separated from water-insoluble alkaloids by a column chromatographic procedure based upon the findings of Levine (6) and Banes (7). Relative concentrations of ergonovine and ergonovinine in the water-soluble alkaloids fraction are estimated by means of a paper chromatographic procedure adapted from that described by Levine and Fischbach (8).

The classical chromogenic reagent, *p*-dimethylaminobenzaldehyde (9), has been employed for the colorimetric assay of ergot alkaloids since its use was first proposed by Smith (10). Burn (11) has suggested that the temperature be controlled carefully during addition of the *p*-dimethylaminobenzaldehyde solution in the determination of ergot alkaloids. When two volumes of *p*-dimethylaminobenzaldehyde T. S. are mixed with one volume of an aqueous solution of an ergot alkaloid, the temperature rises rapidly, and the intensity of the blue color developed in replicate tests is variable. If the flask is swirled continually during slow dropwise addition of the reagent to the alkaloidal solution, the color intensity may be increased by more than ten per cent. Reproducibility is improved and maximum colors are developed when the flask is cooled in an ice bath during dropwise addition of the reagent. (This was especially true in the presence of materials which may char, such as sugars.) These precautions were observed in devising the proposed method.

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ASSAY PROCEDURE

The whole procedure, including paper chromatography but excluding the assay of total alkaloids, must be carried out on the same day and in subdued light.

Extracting Solvent.—Dissolve 10 ml. of ammonium hydroxide, reagent grade, in 90 ml. of methyl alcohol and mix with 900 ml. of chloroform.

Color Developing Reagent.—U. S. P. XVI *p*-dimethylaminobenzaldehyde T. S. (12).

Standard Solution.—Weigh 6.78 mg. of ergonovine maleate U. S. P., dissolve in 1% tartaric acid solution, and dilute to 250 ml. with water. This solution contains the equivalent of 20 mcg. ergonovine per ml.

Diatomaceous Silica Support.—Boil 150 Gm. chromatographic siliceous earth[1] with 1 L. hydrochloric acid (1 + 1) for ten minutes and cool. Then wash with water to remove acid and dry in oven at 100°. The washed product should give no test for acid when moistened.

Chromatographic Tube.—Prepare chromatographic tube from 25 × 200-mm. test tube. Fit it with a packing rod about 400 mm. long, having flat head 22–23 mm. in diameter. Place a small wad of glass wool in the bottom of the tube.

Extraction of Alkaloids.—Weigh 5 Gm. of ergot, ground to No. 60 powder, into a 125-ml. separator. Add 50 ml. of extracting solvent and shake vigorously for five minutes. Add 50 ml. of chloroform and 2 ml. of water, shake gently, and allow to settle. Draw off the lower phase as completely as possible, and filter through a plug of glass wool into a 200-ml. volumetric flask. Add another 20 ml. of extracting solvent to the ergot residues in the separator, mix, add 20 ml. of chloroform and shake. Filter the lower phase through the glass wool into the volumetric flask. Repeat rinse procedure once with an

[1] Celite No. 545, Johns-Manville Co.

additional 20 ml. of extracting solvent and 20 ml. of chloroform. Dilute the combined extracts to 200 ml. with chloroform and mix.

Preparation of Total Alkaloid Solution.—Evaporate a 25-ml. aliquot of the chloroform-ammonia extract solution to dryness at a temperature not exceeding 40°. (If desired, dried sample may be stored overnight in a refrigerator for continuation of assay.) Dissolve the residue in 20 ml. of ether and transfer to a 125-ml. separator with the aid of three 20-ml. portions of ether followed by 5-ml. and 3-ml. portions of approximately 0.2 *N* sulfuric acid. (To check for complete transfer, add 2 ml. of acid to flask and examine under ultraviolet light. There should be no fluorescence.) Shake and draw off the acid layer into a 25-ml. volumetric flask through a plug of glass wool. Extract the ether layer with 5-, 5-, and 3-ml. portions of 0.2 *N* sulfuric acid. Combine the acid extract and washings and dilute to 25 ml. This solution is the *Assay Preparation of Total Alkaloids*.

Preparation of Water-Soluble Alkaloid Solution.—Place 5 Gm. of chromatographic siliceous earth and 5 ml. of 0.1 *M* citric acid in a beaker. Mix thoroughly until mixture appears fluffy and uniform, and transfer to the chromatographic tube. Tap side of tube gently to let mixture settle. Press down firmly and evenly with packing rod. Then mix 2 Gm. siliceous earth with 2 ml. of water and pack the mixture on top of the acid trap. Finally place a layer of glass wool on top of the column.

Evaporate a 150-ml. aliquot of the chloroform-ammonia extract to dryness at a temperature not exceeding 40°. There should be no odor of ammonia or of solvent. Dissolve the residue in 10 ml. of chloroform and transfer quantitatively to the acid trap column with the aid of several small portions of chloroform. Wash down the sides of the chromatographic tube with chloroform and then elute water-insoluble alkaloids with 125 ml. of water-saturated chloroform. Inspect for proper retention of water-soluble alkaloids by very briefly holding the column under an ultraviolet lamp. A brightly fluorescent blue ring in the citric acid layer indicates that the ergonovine has been properly retained. Discard eluent and glass wool cover. Extrude column containing sample with very gentle air pressure into a 400-ml. beaker, mix with 5 ml. of 10% sodium bicarbonate, then mix with enough siliceous earth to make a workable mixture and transfer quantitatively to the chromatographic tube. Rinse packing rod and stirrer with a small amount of chloroform into a beaker. Add enough siliceous earth to adsorb the chloroform and transfer to the column. Rinse again with chloroform. Pass 75 ml. of water-saturated chloroform through the column receiving the eluent in a 125-ml. separator containing 5 ml. of approximately 0.2 *N* sulfuric acid. Examine under an ultraviolet lamp to check for complete removal of water-soluble alkaloids from the column. There should be no fluorescence due to ergot alkaloids.

Shake the mixture of acid and eluate, and draw off the chloroform layer into a second separator. (If after standing fifteen minutes, the organic phase does not completely settle out free of emulsion, add about 30 mg. of anhydrous sodium sulfate and shake until it dissolves.) Transfer the acid layer through the mouth of the separator into a 25-ml. volumetric flask. Rinse first separator and then chloroform with 5-ml. portion of approximately 0.2 *N* sulfuric acid and add the rinse to the acid solution in the volumetric flask. Repeat rinse twice. Dilute the combined acid extracts to 25 ml. and mix. This is the *Assay Preparation of Water-Soluble Alkaloids*.

Development of Color.—Transfer 5.0-ml. aliquots of *Assay Preparations* and of *Standard Solution* to separate stoppered Erlenmeyer flasks. Immerse in an ice bath and add, dropwise, 10.0 ml. of Color Developing Reagent with constant swirling. Allow to stand at room temperature for forty-five to sixty minutes with occasional mixing. Determine the absorbance in a suitable spectrophotometer, at 550 mμ, using as reference blank a mixture prepared by adding 10.0 ml. of reagent to 5.0 ml. of 0.2 *N* sulfuric acid.

Calculations.—Total alkaloid content as per cent ergotoxine = $0.150 \times A/S$, where A is the absorbance of the aliquot of *Assay Preparation for Total Alkaloids* and S is absorbance of the Standard. Water-soluble alkaloid content, as per cent ergonovine = $0.01333 \times A/S$, where A is the absorbance of the aliquot of *Assay Preparation of Water-Soluble Alkaloids* and S is the absorbance of the Standard.

PAPER CHROMATOGRAPHIC PROCEDURE

Developing Solvent.—Mix 100 ml. *n*-butyl acetate, 50 ml. nitromethane, 50 ml. ethylene glycol monoethyl ether acetate (Cellosolve acetate), 8 ml. pyridine, and 10 ml. water. Discard aqueous phase.

Immobile Solvent.—Prepare pH 6 sodium hydroxide-potassium phosphate buffer (13).

Standard Solution.—Dissolve 6.78 mg. ergonovine maleate U. S. P. in 2 ml. of alcohol. This solution contains the equivalent of 2.5 mcg. ergonovine per μL.

Chromatographic Tanks.—Assembly apparatus for ascending paper chromatography with 8 × 8 inch sheets.[2] Blotter paper liners must be used. Ascending paper chromatographic techniques and apparatus have been described in detail by Mitchell (14).

Preparation of Sample.—Render alkaline with sodium bicarbonate a 20-ml. portion of *Assay Preparation of Water-Soluble Alkaloids*, and extract alkaloids with four 10-ml. portions of chloroform. Evaporate combined extracts to dryness at a temperature not exceeding 40°. Just before spotting dissolve residue in 0.5 ml. of alcohol.

Procedure.—Equilibrate developing solvent in tank for thirty minutes. Pour onto marked 8 × 8 inch paper about 10 ml. of buffer solution. Blot with paper towels to remove excess moisture. Quickly spot 1, 2, and 5 μL. of standard solution and 5 and 10 μL. of sample solution. Moisten spots with water from a capillary tube. A spot at edge of paper of methyl red indicator in alcohol will aid in locating solvent front. When paper feels moist and limp, but not so wet as to transfer water to the fingers, place in tank. When solvent front is within an inch of the top of the paper, remove and dry in hood. Examine under ultraviolet lamp.

[2] Arthur H. Thomas Co., No. 3677, has been found suitable.

TABLE I.—COMPARISON OF ASSAY METHODS[a]

Method of Assay	% Water-Soluble Alkaloids: Sample No. 2	Sample No. 5	Sample No. 6	Sample No. 9
N. F. percolation and separation	0.0084	0.0100	0.0142	0.0218
N. F. percolation column separation	0.0087	0.0081	0.0114	0.0216
2-Phase extraction N. F. separation	0.0123	0.0125	0.0181	0.0256
2-Phase extraction column separation	0.0123	0.0124	0.0171	0.0279

[a] All assays by the same analyst.

Blue fluorescent spots of ergonovine appear about one-third to one-half the distance from starting line to the solvent front with ergonovinine spots immediately ahead.[3] The water-soluble alkaloids are equally fluorescent, thus a rough estimation of ergonovine and ergonovinine contents can be made by comparing the intensity of fluorescence of various sample spots with those of the standard spots.

RESULTS AND DISCUSSION

The ammonia-methanol-chloroform combination proved the most effective of the extracting solvents compared. Some ergot residues from the N. F. method of percolation were extracted a second time by shaking. As much as 5% of the total alkaloids were extracted from the residues. In the case of the two-phase shaking method no more than 0.5% of total alkaloids or 1% of the water-soluble alkaloids were ever recovered in a second extraction. The variation in collaborative results reported by Strong and Maurina (2) may be due in part to the inefficiency of the single-phase extraction process. The inadequacy of the percolation method is further indicated in Table I which compares the two methods of extraction. Table I also shows that the N. F. and chromatographic column methods for the isolation of water-soluble alkaloids yield similar results.

The findings recently reported by Vining and Tabor (15) corroborate our conclusion that a two-phase system is necessary for extraction. These authors report that ergot alkaloids were best extracted from lyophilized saprophytic cultures of *Claviceps purpurea* with a small amount (1 ml.) of 10% ammonia solution and ether (50 ml.) or chloroform. They state that "wetting of the culture was necessary to release the alkaloid from adsorptive binding to the culture solids since none could be extracted with dry nonpolar solvents."

The 0.1 *M* citric acid trap was found to retain ergonovine and ergonovinine quantitatively. Ergotamine was quantitatively eluted from the citric acid trap with 70 ml. of chloroform and ergotoxine with 60 ml. To check recoveries of ergonovine, aliquots of a solution of ergonovine maleate U. S. P.

TABLE II.—COMPARISON OF RESULTS OBTAINED BY SEVERAL ANALYSTS[a]

Analyst	Sample No. 3: % Total Alkaloids	Sample No. 3: % Water-Soluble Alkaloids	Sample No. 7: % Total Alkaloids	Sample No. 7: % Water-Soluble Alkaloids
1	0.188	0.0160	0.201	0.0204
	0.188	0.0162	0.206	0.0208
	0.195	0.0163	...	0.0208
	0.195	0.0163	...	0.0208
	...	0.0164	...	0.0211
	...	0.0167	...	0.0213
	...	0.0170	...	...
2	0.180	0.0159	0.193	0.0204
	0.181	0.0163	...	...
3	0.187	0.0161	...	...
	0.188	0.0161	...	...
4	0.176	0.0163	0.185	0.0209
	0.176	0.0164	0.186	0.0210
Average	0.185	0.0163	0.194	0.0208

[a] Each pair of figures represents a separately weighed and analyzed portion of ergot. The authors are indebted to the following for their help in this collaborative study: J. Wolff, Div. of Pharm. Chem., Food and Drug Admin.; S. Cohen, Minneapolis District, Food and Drug Admin.; J. Salas, Caracas, Venezuela.

[3] If the spots diffused badly, it indicates that paper was too wet when placed in tank. If spots did not move, paper probably was too dry.

TABLE III.—COMPARISON OF RESULTS OBTAINED USING N. F. AND PROPOSED PROCEDURES[a]

Sample Number	N. F. Method: % Total Alkaloids	N. F. Method: % Water-Soluble Alkaloids	Proposed Method: % Total Alkaloids	Proposed Method: % Water-Soluble Alkaloids
1	0.201	0.0197	0.224	0.0264
	0.189	0.0198	0.228	0.0262
2	0.156	0.0081	0.163	0.0117
	0.160	0.0084	0.166	0.0136
			0.158	0.0126
			0.177	0.0125
			0.182	0.0124
3	0.204	0.0160	0.188	0.0163
	0.209	0.0166	0.188	0.0163
	0.207	0.0166	0.195	0.0170
	0.202	0.0152	0.195	0.0164
	0.191	0.0158	...	0.0167
			...	0.0160
			...	0.0162
4	0.148	0.0089	0.178	0.0119
	.146	0.0092	0.158	0.0137
	.140	0.0099	0.163	0.0136
			0.162	0.0137
5	0.129	0.0100	0.150	0.0121
			0.140	0.0127
6	0.161	0.0142	0.190	0.0169
			0.185	0.0173
7	0.210	0.0231	0.201	0.0213
	0.212	0.0205	0.206	0.0204
	0.217	0.0198	...	0.0211
	0.202	0.0192	...	0.0208
	0.202	0.0194	...	0.0208
			...	0.0208
8	0.202	0.0224	0.237	0.0284
			0.228	0.0286
9	0.215	0.0218	0.230	0.0286
	0.218	0.0211	0.233	0.0286
	0.201	0.0218	0.236	0.0276
			0.229	0.0279
			0.234	0.0280
			...	0.0281

[a] Each pair of figures represents a separately weighed and analyzed portion of ergot. All assays by the same analyst.

were rendered alkaline with sodium bicarbonate, mixed with siliceous earth, and packed in a chromatographic tube. Alkaloid was eluted with chloroform and analyzed by the proposed column separation method. Recoveries ranged from 95 to 98%. In similar experiments, when ergotamine and ergotoxine salts were employed, quantities of alkaloid equivalent to not more than 3% of the ergonovine were found in the water-soluble fraction. These two sets of errors tend to balance each other.

Table II shows the results obtained by several different analysts when using the proposed two-phase shaking extraction and column separation procedure. Agreement between analysts was satisfactory. Several commercial samples of official rye ergot were analyzed by the same analyst using both the N. F. and proposed procedures. The results of these analyses are compiled in Table III. Higher assay results for water-soluble alkaloids invariably were obtained by the proposed procedure. The N. F. requires that the total alkaloid content be not less than 0.15% and that the water-soluble alkaloid content be not less than 0.01%. Samples 2, 4, and 5 do not meet these specifications when analyzed by the N. F. method. All samples meet these minimum specifications when analyzed by the proposed procedure.

The chloroform extracts of several commercial samples were chromatographed on paper. In all cases, the ergonovine spot was more intensely fluorescent than was the ergonovinine spot. The sum of the ergonovine and ergonovinine contents, in some cases, did not seem to equal that of water-soluble alkaloid content by assay. It is assumed that various alkaloid breakdown products are included in the water-soluble alkaloid fraction. Under extraordinary conditions, such as marked changes in temperature from that of ordinary operation, poor resolution may be encountered due to excessive or insufficient movement of the alkaloids. The R_f's should be in the range of 0.2–0.8. If higher R_f's are desired, a less acidic buffer should be employed.

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Chemical Investigation of *Dioscorea macrostachya* (Benth.)

By WILLIAM A. MEER† and PAUL J. JANNKE

The ultimate analysis of lipoidal matter extracted from the roots and tubers of *Dioscorea macrostachya* (Benth.) disclosed fatty acids present to be palmitic, stearic, oleic, and linoleic. The unsaponifiable matter yielded β-sitosterol, and the presence of unsaturated hydrocarbons, a wax and yellow pigmentary material was indicated. A sterol glycoside isolated was shown to be β-sitosterol glucoside. Proximate analysis for group constituents was also run.

MAJOR INTEREST in the genus *Dioscorea* during recent years centers about its potential as a source of cortisone-like compounds. Since 1943, several hundred species have been examined for their steroidal sapogenin content by Marker, *et al.* (1), and by Wall and his associates (2, 3). Some of the reports of the latter group include qualitative tests for flavanols, alkaloids, tannins, sterols, organic acids, and phenols. However, there is very little else in the literature on the chemical composition of *Dioscorea* spp. in general and none on *D. macrostachya* in particular. This report deals with a proximate analysis of the constituents of the tubers of this species and with an ultimate analysis of the lipoidal matter isolated from the tubers.

The plant material used in the investigation was obtained from the San Pedro Sula region of Honduras. In 1957, 55 pounds of roots and

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† Present address: Meer Corp., 318 W. 46th St., New York 36, N. Y.

tubers were imported through the cooperation of the Eastern Utilization Research Branch and the Horticultural Crops Research Branch of the United States Department of Agriculture, Beltsville, Md.[1] The shipment was received, inspected, and fumigated (methyl bromide) at the Plant Quarantine Branch of the Agricultural Research Service in Washington, D. C.

EXPERIMENTAL

Proximate Analysis of the Plant Material

Approximately 50 pounds of plant material was dried for one week at 50° in a circulating hot air oven whereupon it was comminuted in a Fitzpatrick mill. The moisture content, determined by the toluene distillation method, was 5.67%. The total ash content was 1.13% and the acid-insoluble ash amounted to 0.24%.

The micro procedure applied by Wall, *et al.* (4), in their screening studies was applied to duplicate 50-Gm. samples of plant material with the results given in Table I in addition to the following: alcohol-soluble extract, 28.42%; water-soluble extract, 12.75%; chloroform-soluble extract, 0.29%.

TABLE I.—PROXIMATE ANALYSIS OF *Dioscorea macrostachya* TUBERS

Test For	Extract Used	Result	Test or Reference
Saponins	Alcohol	+	Bamford (5)
Flavanols	Alcohol	−	Bryant (6)
Alkaloids	Aqueous	−	Mayer's and Wagner's
Tannins	Aqueous	+	Wilson (7)
Organic acids and phenols	Aqueous	+	Wall, *et al.*
Reducing sugars	Aqueous		
Before hydrolysis		−	Fehling's
After hydrolysis		+	Fehling's
Sterols	Chloroform		
Unsaturated		+	Liebermann-Burchard (8)
Conjugated unsaturated		−	Rosenheim (9)

Ultimate Analysis of the Lipids from the Tubers

Two portions of the dried, comminuted plant material, weighing 10.9 Kg. and 6.8 Kg. each, were extracted with low boiling petroleum ether in a Lloyd extractor. After recovery of the solvent under reduced pressure and under an atmosphere of carbon dioxide, the yield of lipoidal extracts amounted to 19.6 Gm. and 16.9 Gm., respectively, corresponding to an overall yield of 0.21%. The saponification value was 123.9 and the iodine number, determined by the micro method of Ralls (10), was 72.82.

A 13.6-Gm. portion of the fatty extract was saponified by refluxing for two and one-half hours with 150 ml. of 3% alcoholic potassium hydroxide. After recovering the alcohol and dissolving the residue in 200 ml. of water, the unsaponifiable matter was extracted with ethyl ether. The ethereal solution was washed with water, dried over anhydrous sodium sulfate, and reserved for later investigation.

The aqueous soap solution was acidified with 10% sulfuric acid and heated, whereupon the liberated fatty acids formed a supernatant layer. After cooling, this was separated mechanically and the acidic aqueous solution was extracted with several small portions of ether. The solidified fatty acids were dissolved in the combined ethereal extracts and the whole was washed with water until the washings were neutral. Recovery of the solvent from the dried ethereal solution left a residue of total fatty acids weighing 10.05 Gm.

A small quantity of solid matter appeared at the aqueous-ethereal interface during the extraction of the fatty acids. It was filtered from the aqueous solution, washed, and dried. Subsequent work showed it to be a sterol glycoside. Normally, this should have appeared at the interface during the extraction of the unsaponifiable matter, but due to the small amount present (0.113 Gm.) it remained suspended in the concentrated soap solution.

Separation and Isolation of the Fatty Acids.—The total fatty acids (weighing 9.452 Gm. when completely dried) were separated by the lead salt-ether method, yielding 3.767 Gm. of saturated acids and 5.685 Gm. of unsaturated acids, corresponding to 39.86% and 60.14%, respectively.

Saturated Fatty Acids.—The saturated fatty acids were converted to the methyl esters by the usual procedure, whereupon 3.667 Gm. of esters were obtained. They were fractionated at reduced pressure on a Todd column (1.5 mm. monel packing), yielding two fractions and a residue.

Fraction I.—This fraction weighed 1.963 Gm., corresponding to 57.73%; n_D^{45} was 1.4322; b. p. at 5 mm. was 165.5–167°. Its saponification equivalent of 270.99 together with the physical properties coincide with those of methyl palmitate. The identity of the ester was further established by converting it to palmitohydroxamic acid, m. p. 102–102.5°.

Fraction II.—The second fraction (1.308 Gm. or 38.47%) proved to be methyl stearate, b. p. 182–184° at 5 mm.; n_D^{45} was 1.4358; saponification equivalent was 294.33. Stearohydroxamic acid prepared from the ester melted at 105–106°.

Residue.—The residue, 0.129 Gm., corresponding to 3.79%, was shown by its saponification equivalent to be a mixture of methyl palmitate and methyl stearate. The quantity was too small to drive through the column without the aid of a chaser.

Unsaturated Fatty Acids.—A sample of the mixed unsaturated fatty acids weighing 0.914 Gm. was brominated in the usual manner. The ethereal solution of the reaction mixture yielded no crystals after being refrigerated, indicating the absence of hexabromolinolenic acid.

After changing to a petroleum ether solvent, crystals were deposited from a cold solution, in a yield of 0.399 Gm. Following recrystallization from the hydrocarbon solvent, the melting point was 115°, which agrees with the constant reported for tetrabromolinoleic acid.

The residue from the mother liquor was debrominated in the usual manner, yielding 0.7216

[1] The authors are grateful for the assistance received from Dr. Quentin Jones, Botanist, Horticultural Crops Research Branch, U.S.D.A., Beltsville, Md.

Gm. of oleic acid, identified as such by its neutralization equivalent of 282.74; n_D^{25} was 1.4734; iodine number, 86.11. Application of the elaidin test converted it to elaidic acid, the transisomer of oleic acid.

Thus, the unsaturated fatty acids were shown to consist of 78.95% linoleic acid and 20.43% oleic acid.

The composition of the unsaturated fatty acid mixture was verified by application of the low temperature chromatographic technique originally described by DiModica and Rossi (11) and modified by DeCastro and Jannke (12). Through this method, involving frontal analysis, only oleic and linoleic acids were shown to be present in the eluates by virtue of their iodine numbers. This substantiated the qualitative results obtained from the bromination procedure.

Sterol Glycoside.—During the ether extraction of the fatty acids, a brown, insoluble residue was obtained. The impure compound decomposed at 255°, and although insoluble in chloroform, it gave a positive Liebermann-Burchard test. Following the hydrolysis of a small quantity with 10% hydrochloric acid, a positive test for reducing sugars was obtained with Fehling's solution. The compound was repeatedly recrystallized from a pyridine-ethanol solvent until it was obtained in the form of flaky white crystals (24.6 mg.) which melted with decomposition at 287–287.5°.

The sterol glycoside was hydrolyzed according to the method of Power and Salway (13). After recovering the volatile solvents, the aqueous liquid was filtered, whereupon a white residue giving a positive Liebermann-Burchard reaction was obtained. After three recrystallizations from hot ethanol, 10 mg. of white, lustrous crystals melting at 136–136.5°, were obtained. This was later shown to be β-sitosterol.

The aqueous acidic filtrate was neutralized with sodium carbonate and evaporated to dryness. The residue was extracted with absolute ethanol and upon evaporation of the solvent, 5 mg. of a semisolid mass was obtained. Three milligrams of this syrupy material was used to prepare an osazone. After immersion in a boiling water bath for fifty-four minutes, the reaction mixture yielded a flocculent precipitate. This time factor compares reasonably with that (forty-seven minutes) reported by Garard and Sherman (14) in their preparation of glucosazone from 5 mg. of dextrose. The osazone melted at 205°, following recrystallization from hot 60% ethanol. A mixed melting point with glucosazone prepared from dextrose was undepressed.

Table II.—R_f Values of Sugar Hydrolysate of Sterol Glycoside[a]

	Solvent			
	90% Phenol[b] Saturated with Water		n-Butanol-Acetic Acid-Water (4:1:5 v/v)	
	I	II	I	II
Sugar hydrolysate	0.39	0.40	0.14	0.15
Glucose	0.39	0.40	0.15	0.15
Arabinose	0.53	0.55	0.19	0.21
Xylose	0.46	0.46	0.22	0.22

[a] R_f values are given for known reducing sugars run on the same chromatograms.

[b] In runs with phenol, 1% ammonia w/v and a few crystals of potassium cyanide were added to the aqueous solution in the bottom of the chamber.

The identity of the sugar moiety of the sterol glycoside was further established by paper partition chromatography, applying the method of Partridge (15, 16). Identification of the unknown sugar was based on the R_f values of authentic specimens of glucose, arabinose, and xylose when run side by side on the same filter paper strip. The unknown sugar appeared alongside glucose in duplicate runs involving two different solvent systems. There was no indication of any other unknown sugar on the chromatograms. Results of the R_f values appear in Table II.

Unsaponifiable Matter.—The combined ethereal solutions of the unsaponifiable matter were washed free of alkali and dried over anhydrous sodium sulfate. Recovery of the solvent left a yellow semisolid residue weighing 5.316 Gm.

In applying a chromatographic separation procedure (12), the entire residue was dissolved in a minimum amount of petroleum ether (65–110°) and adsorbed on a 4.5 cm. × 35 cm. column of Fisher adsorption alumina. The column was successively eluted with 2 L. of petroleum ether, 5 L. of carbon tetrachloride, 3.5 L. of benzene, and 2 L. of chloroform containing 10% absolute ethanol, and 1 L. of chloroform containing 20% of absolute ethanol. The volumes used in each case represent complete exhaustion of the column fractions soluble in the particular solvent employed. The extracts were freed of their respective solvents, dried under vacuum, and weighed. The results are given in Table III.

Table III.—Chromatography of Unsaponifiable Matter

Eluents	Recovery, Gm.	Liebermann-Burchard Test
Petroleum ether	2.2262	−
Carbon tetrachloride	0.4884	−
Benzene	0.1534	−
Chloroform-ethanol	2.4109	+
Total	5.2789[a]	

[a] Original sample weighed 5.3163 Gm.

Petroleum Ether Eluate.—The residue remaining after removal of the solvent was a colorless to light yellow liquid which solidified when cooled slightly below room temperature. Hydrocarbons are reported to occur in this fraction. The residue gave no color reaction in the Liebermann-Burchard test, but it reacted with both concentrated sulfuric acid and dimethyl sulfate and it decolorized a weak solution of bromine in carbon tetrachloride. Its specific gravity at 25° was 0.8888; $n_D^{40} = 1.4604$. Attempts to prepare a solid alkyl acid sulfate and a solid bromine adduct were not successful. The physical constants recorded do not apply to any particular unsaturated hydrocarbon. It was therefore concluded to be a mixture.

Carbon Tetrachloride Eluate.—This eluate yielded a dark brown semisolid mass which was insoluble in alcohol and which gave a negative Liebermann-Burchard test. A positive reaction to Feigl's test (17) for carboxylic acid esters was observed. De Castro and Jannke (12) have shown that this solvent elutes only the waxes from the unsaponifiable matter. The material was only partially hydrolyzed by digestion with amyl alcohol and 30% hydrochloric

acid, nevertheless the presence of a carboxylic acid was demonstrated by the reaction of ferric chloride with the acid hydroxamate. Attempts to prepare the phenacyl bromide were not successful. The alcohol liberated by the hydrolysis did not yield a phenyl urethan. The quantity of acid and alcohol available at this point was too small to permit further investigation.

Benzene Eluate.—This portion of the unsaponifiable matter eluted with benzene was of a deep orange color, suggestive of the carotenoid pigments normally found in some fats. Zechmeister and Cholnoky (18) reported the isolation of lycopene, lycoxanthin, and lycophyll, in crystalline form, from the fruits and seeds of a member of the genus, Dioscorea.

The residue was purified by the method described (19) for the carotenes. Elution of the chromatographic column under vacuum and subsequent removal of the solvent yielded 0.141 Gm. of residue. Material retained on the column was eluted with chloroform. It weighed 0.013 Gm. Chloroformic solutions of both fractions were analyzed spectrophotometrically (Beckman model B). Standard β-carotene run as a control have absorption maxima at 465 mμ and 598 mμ, but no peaks were observed for either of the fractions tested. Their absorption curves, however, indicated them to be the same substance.

The residues dissolved in chloroform did not give characteristic color reactions for β-carotene when treated with antimony chloride. They did not respond to acetylation and benzoylation, indicating the absence of hydroxyl functions. The pigment obtained may have been a degradation product of carotene.

Chloroform—Ethanol Eluates.—Elution with chloroform-10% ethanol gave residues responding very strongly to the Liebermann-Burchard test for sterols. A more polar solvent containing 20% ethanol was used to strip the column of sterol. After the solvent was removed from the combined fractions, the yellowish crystalline residue was taken up in 95% ethanol and decolorized by refluxing with Norite A. Repeated recrystallization of the product from ethanol yielded lustrous white platelets which melted at 136–136.5°. A mixed melting point with β-sitosterol was undepressed. Its acetate and benzoate prepared in the usual manner likewise did not depress the melting point of the corresponding esters of β-sitosterol. The specific rotation was determined on a solution of 200 mg. of the sterol in 2 ml. of chloroform. $[\alpha]_D^{27} = -38.2°$. The constant recorded for β-sitosterol is $-37.0°$.

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Studies on the Estimation of Folic Acid in Multivitamin-Mineral Preparations

By O. PELLETIER and J. A. CAMPBELL

The permanganate oxidation method of Schiaffino, *et al.*, was applied to known mixtures of folic acid with iron salts, ascorbic acid, and other constituents of multivitamin-mineral preparations. Some difficulty was experienced in the recovery of folic acid from these preparations. The use of a standard blank absorbance reading improved the accuracy of recovery tests. Interference of ferrous sulfate was eliminated by ascorbic acid and that of ferric ammonium citrate by chelating with EDTA. Interference caused by red dyes in certain preparations and by some pigments in biological extracts was not completely removed by isobutyl alcohol extraction of the folic acid reaction product but could be eliminated by the use of appropriate blank readings. Low recovery of folic acid in the presence of some multivitamin-mineral mixtures was overcome by the use of a trisodium phosphate-calcium lactate extracting medium. With this extractant, ferrous gluconate caused interference but this was eliminated by the use of EDTA. Folic acid was more effectively removed and more stable in the phosphate-lactate extractant than in the potassium phosphate solution. *para*-Aminobenzoic acid, which interfered with the reaction, was extracted with ethyl acetate. The proposed modification yielded complete recovery of folic acid.

THE CHEMICAL determination of folic acid using the Bratton and Marshall reagent (1) has been improved recently by Schiaffino, *et al.* (2), who recommended permanganate oxidation in place of zinc reduction as outlined in U. S. P. XV (3). Their procedure involved extracting the folic acid in a mildly alkaline buffered solution (3 per cent potassium phosphate), splitting it with potassium permanganate, diazotizing the free amine, and coupling with the Bratton and Marshall reagent. The final colored product was extracted with isobutyl alcohol when interfering colors were present.

In this laboratory an attempt was made to eliminate the effect of substances in pharmaceutical preparations which interfered with the zinc reduction method. The use of 4 per cent trisodium phosphate as an extractant as suggested by Shah, *et al.* (4), yielded slightly low recoveries of folic acid in the presence of ferrous salts and did not precipitate ferric salts. If calcium lactate were added to the phosphate solution good recovery of folic acid was obtained. Since difficulty had been experienced in preliminary tests of some samples by the method of Schiaffino, *et al.*, it seemed appropriate to incorporate the phosphate-lactate extractant into the permanganate procedure and to investigate this reaction in more detail. Optimum conditions for the reaction were determined with the new extractant. Use was also made of ethylenediaminetetraacetic acid (EDTA) as suggested by Shah, *et al.* (4), for the complexometric masking of ferrous gluconate in the assay solution.

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This paper describes a modified method and demonstrates how complete recovery of folic acid can be obtained by this procedure and by modifications of that of Schiaffino, *et al.*

METHOD

Reagents.—Potassium phosphate solution (dibasic) 3%, dissolve 30 Gm. of reagent grade anhydrous K_2HPO_4 to make 1,000 ml. Trisodium phosphate, 8%, dissolve 80 Gm. of $Na_3PO_4 \cdot 12H_2O$ in water to make 1,000 ml. Calcium lactate, 3%, dissolve 30 Gm. of calcium lactate in water to make 1,000 ml.; warm to dissolve completely. Lactate-phosphate mixture, mix equal volumes of 8% trisodium phosphate and 3% calcium lactate as necessary before each assay. Metaphosphoric acid, 2%, dissolve 2 Gm. HPO_3 in water to make 100 ml.; prepare fresh daily. Potassium permanganate, 4%, dissolve 20 Gm. of reagent grade $KMnO_4$ in water to make 500 ml. Potassium permanganate, 0.4%, dilute 10 ml. of the 4% $KMnO_4$ with water to make 100 ml.; prepare fresh solution for each assay. Sodium nitrite, 2%, dissolve 2 Gm. of reagent grade $NaNO_2$ in water to make 100 ml.; prepare fresh daily. Hydrochloric acid, 5 *N*, dilute 445 ml. of reagent grade (35–37%) HCl with water to make 1,000 ml. Ammonium sulfamate, 5%, dissolve 5 Gm. of reagent grade $NH_4SO_3 \cdot NH_2$ in water to make 100 ml. EDTA solution, 5%, dissolve 5 Gm. of ethylenediaminetetraacetic acid in about 25 ml. of water with 1 *N* sodium hydroxide as required; make up to 100 ml. with water. N-(1-Naphthyl)-ethylenediamine dihydrochloride, 0.1% (NED solution), dissolve 100 mg. of reagent grade N-(1-naphthyl)-ethylenediamine dihydrochloride in water to make 100 ml.; prepare fresh solution for each assay. Sodium chloride, reagent grade NaCl. Isobutyl alcohol, grade suitable for spectrophotometric and fluorometric use.

Folic Acid Standard Solutions.—*Stock Solution.*—Weigh accurately, in a closed system, U. S. P. Folic Acid Reference Standard, equivalent to 50–60 mg. of folic acid, that has been dried to a constant

weight and stored in the dark over phosphorus pentoxide in a desiccator. Dissolve in about 50 ml. of 0.4% ammonia solution, and dilute with water to make the folic acid concentration exactly 500 mg. per ml. Store under toluene in the dark at about 10°.

Standard Solution.—Dilute 2 ml. of the Folic Acid Stock Solution with the 3% potassium phosphate solution to make 100 ml. Each ml. contains 10 mcg. of folic acid. Designate this as the Standard Solution. Prepare fresh Standard Solution for each assay.

Assay Solution.—Avoid undue exposure to light of samples containing riboflavin. Place a measured amount of sample in a volumetric flask, mix, and make to volume with lactate-phosphate extractant. The volume of lactate-phosphate extractant should not be less in milliliters than 10 times the dry weight of the sample and the resulting solution must contain not more than 0.1 mg. folic acid per ml. If sample is not readily soluble, comminute so that it may be evenly dispersed in the liquid. (It may be desirable to remove tablet coatings or capsule shells containing red dyes prior to extraction.) Heat the mixture to about 50° while shaking vigorously until a good dispersion is obtained. Centrifuge about 45 ml., let cool, and dilute an aliquot from the supernatant solution with lactate-phosphate extractant to obtain a final concentration of 5 mcg. per ml. Filter, discarding the first few ml. of filtrate, and designate as Assay Solution.

Assay Tubes.—To each of eight tubes or reaction vessels, 40-ml. size, add 2.0 ml. of Assay Solution. Designate and prepare the tubes as follows: *Tubes A.*—To each of two of the tubes add 3 ml. of 3% K_2HPO_4 solution and mix. *Tubes B.*—To each of two of the tubes add 1 ml. of the Standard Solution and 2 ml. of the 3% K_2HPO_4 solution and mix. *Tubes C.*—Prepare as for tubes A. *Tubes D.*—Prepare as for tubes B.

Add 1 ml. of 2% metaphosphoric acid to all tubes except when samples contain ferrous salts in the absence of easily oxidizable substances such as ascorbic acid. To each tube designated A and B, add, with mixing, 1 ml. of the 0.4% $KMnO_4$ solution and allow to stand two to three minutes. During the two- to three-minute oxidation, the solution should retain the purple permanganate color since metaphosphoric acid usually prevents its decolorization by highly oxidizable material; if the color fades, increase the quantity of $KMnO_4$ until there is no further decolorization. To each of the remaining tubes add 1 ml. of water and mix. Whenever the volume of $KMnO_4$ solution is increased, add an equivalent volume of water to the remaining tubes. To all tubes add 1 ml. of the 2% $NaNO_2$ solution and mix; followed by 1 ml. of the 5 *N* HCl solution, and mix. Allow to stand two minutes; then to each tube add 1 ml. of the 5% $NH_4SO_3 \cdot NH_2$ solution, and mix. Swirl tubes vigorously at intervals for about three minutes until excess nitrogen dioxide is expelled. (Excess of nitrogen dioxide will interfere with color development.) If the sample contains ferrous gluconate add to each tube designated A, B, C, and D, 1 ml. of EDTA solution. To all tubes add 1 ml. of the 0.1% N-(1-naphthyl)-ethylenediamine dihydrochloride (NED) solution, mix, and allow to stand ten minutes for maximal color development. For assay solution that contains red dyes or other interfering pigments which are decolorized with $KMnO_4$ prepare and designate four additional tubes as follows: *Tubes X.*—Prepare two tubes as for tube A but replace the NED reagent by 1 ml. of water. *Tubes Y.*—Prepare tubes as for tubes C but replace the NED reagent by 1 ml. of water. Proceed as directed under (*a*) or (*b*):

(*a*) *For Assay Tubes That Contain No Interfering Material.*—Within ten to twenty-five minutes after addition of NED, using water as the "blank," measure the absorbance of the solution from each tube as directed under Determination.

(*b*) *For Assay Tubes That Are Cloudy Or Dark Colored In An Amount That Interferes with Final Color Measurements.*—Add to each tube 1 Gm. of sodium chloride and 10 ml. of isobutyl alcohol, and shake vigorously two or three minutes. Centrifuge tubes until supernatant isobutyl alcohol solution is clear. Using isobutyl alcohol as the "blank," measure the absorbance of the isobutyl alcohol solution from each tube as directed under Determination.

Determination.—Measure the absorbance of the solution from each tube at 545 mμ in a suitable photoelectric colorimeter set at 0 absorbance with the "blank."

Calculation.—Calculate on basis of aliquots taken as follows, using the absorbances *A*, *B*, *C*, and *D*. (If the color blanks are required, correct *A* and *B* by subtracting *X* from each, and correct *C* and *D* by subtracting *Y* from each.)

$$\text{mg. of folic acid in sample} = \frac{A - C}{(B - A) - (D - C)} \times 0.005 \times \text{dilution factor}$$[1]

Modification of Method for Samples Containing *p*-Aminobenzoic Acid (PAB).—Remove PAB by extraction with 5% benzoic acid in ethyl acetate (w/v) as follows: if concentration of original sample is less than 100 mcg. per ml., use solid reagents to maintain proper pH and a concentration of 4% $Na_2PO_4 \cdot 12\,H_2O$ and 1.5% calcium lactate by weight. To each of two 50-ml. centrifuge tubes add 5 ml. assay solution containing 20 mcg. per ml. and 10 ml. of 2% metaphosphoric acid and mix. To tube No. 1, add 1 ml. of 3% K_2HPO_4 and mix. To tube No. 2, add 1 ml. of standard solution containing 100 mcg. and mix. To each tube add approximately 20 ml. of ethyl acetate-benzoic acid solution and shake vigorously for about two minutes. Centrifuge and carefully syphon off most of the top layer without removing any of the bottom layer. Repeat ethyl acetate-benzoic acid extraction twice in the same manner and transfer quantitatively the aqueous layer to a 50-ml. volumetric flask, using 15 ml. of filtered lactate-phosphate solution. Make to volume with 3% K_2HPO_4. For tubes A and C add 5 ml. of the diluted solution from tube No. 1 above. For tubes B and D add 5 ml. of the diluted solution

[1] $\frac{A - C}{(B - A) - (D - C)}$ represents the ratio, sample folic acid/standard folic acid, which is more accurate than $\frac{A - C}{B - A}$ which represents the ratio, sample folic acid/(standard folic acid + standard free amines) where A = sample folic acid + sample free amines; B = sample folic acid + sample free amines and standard folic acid + standard free amines, C = sample free amines; and D = sample free amines and standard free amines.

Table I.—Per Cent Recoveries[a] of Folic Acid in Presence of Ascorbic Acid and Ferrous Salts Alone and Together

Substance and Ratio		Schiaffino, *et al.*, Method: Without Std. Blank	Schiaffino, *et al.*, Method: With Std. Blank	Proposed Method
Nil	Nil	97.3	100.0	100.3
Ascorbic acid	100	95.0	98.5	100.6
Ascorbic acid	150	94.7	98.0	100.0
Ascorbic acid	200	93.5	99.1	99.4
Ascorbic acid	300	94.5	98.1	100.9
Ascorbic acid	400	94.3	99.5	100.8
Ascorbic acid	500	92.2	98.4	100.3
Ferrous gluconate	600	96.2	98.9	99.2
Ferrous gluconate	1200	96.4	99.5	98.0
Ferrous gluconate + ascorbic acid	600 150	94.9	98.3	99.4
Ferrous sulfate	125	84.3	86.5	99.5
Ferrous sulfate	250	86.3	88.1	100.3
Ferrous sulfate	500	84.0	86.7	99.0
Ferrous sulfate + ascorbic acid	250 150	94.3	98.3	99.1

[a] Each value is the mean of two separate assays.

from tube No. 2 above. Add 1 ml. of 3% K_2HPO_4 to all tubes and proceed as in the regular method with the addition of 1 ml. of the 0.4% $KMnO_4$ solution. For calculation use factor of 0.02 in place of 0.005.

RESULTS

Absorption Maximum of Reaction Product.—The absorption maximum of the color produced in this reaction determined at several concentrations of folic acid on a Spectracord recording spectrophotometer was 545 mμ. In the studies reported below, a 540 filter was used in the Evelyn colorimeter.

Effect of Ascorbic Acid and Iron Salts, Alone and in Combination, on Recovery of Folic Acid.—Mixtures of known amounts of U. S. P. Reference Standard Folic Acid with ascorbic acid and/or iron salts were assayed by both the method of Schiaffino, *et al.*, with and without a standard blank, and by the method described above. The amount of folic acid used for each assay was 0.5 or 1 mg. and the proportions of ascorbic acid and iron salts are given in Table I along with the results. At all proportions of ascorbic acid recoveries were satisfactory by the former method using a standard blank and also by the modified method. It was necessary to use additional $KMnO_4$ at the higher levels. Relatively high proportions of ferrous gluconate, with or without ascorbic acid, caused practically no loss of folic acid by Schiaffino's procedure using a standard blank or by the proposed procedure where 1 ml. of 5% EDTA solution was added before the Bratton and Marshall reagent to prevent the formation of a precipitate. Values obtained without the use of the standard blank reading were consistently lower than when this reading was used. As also shown in Table I, low recoveries of folic acid were caused by the presence of ferrous sulfate in Schiaffino's procedure, but the presence of ascorbic acid in addition to ferrous sulfate produced higher recoveries.

To explain the low results obtained by the original method in the presence of ferrous sulfate, the absorbances used to calculate these data are presented in Table II. In this method the absorbances (total) due to folic acid and free amines were consistently lower for the assay solution than for the corresponding increment of standard solution, and the absorbances (blank) due to the free amines alone were markedly higher for the assay solution than for the standard solution increment, although both solutions were from the same stock solution of folic acid. These results indicated that the presence of ferrous sulfate in the assay solution caused a loss of folic acid and a corresponding increase in free amines. In the proposed method, absorbances were similar for the assay solution and for the standard increment solution, indicating complete recovery of folic acid.

Recovery tests of folic acid in the presence of ferric ammonium citrate are shown in Table III. The method of Schiaffino, *et al.*, was carried out, both with and without isobutyl alcohol extraction, and also modified by the use of 1 ml. 5% EDTA before adding the Bratton and Marshall reagent to prevent the formation of a greenish color with ferric ammonium citrate. With the Schiaffino method, satisfactory recoveries were obtained up to proportions of ferric ammonium citrate to folic acid of 200:1 without isobutyl alcohol extraction, 400:1 with isobutyl alcohol extraction, and 800:1 using EDTA. When 150 parts of ascorbic acid were included with 400 of ferric ammonium citrate, this method gave satisfactory results only when EDTA was used. The use of a standard blank improved recoveries slightly with EDTA but had no effect in the absence of this reagent. With the proposed method there was no indication of interference from ferric ammonium citrate with or without ascorbic acid.

Table II.—Absorbances of Assay Solution and Increment of Standard Solution in Presence of Increasing Proportion of Ferrous Sulfate

Ratio $FeSO_4$/ Folic Acid	Schiaffino, *et al.*, Method: Assay Solution: Total Tube A	Schiaffino, *et al.*, Method: Assay Solution: Blank Tube C	Schiaffino, *et al.*, Method: Standard Increment: Total B-C	Schiaffino, *et al.*, Method: Standard Increment: Blank D-C	Proposed Method: Assay Solution: Total Tube A	Proposed Method: Assay Solution: Blank Tube C	Proposed Method: Standard Increment: Total B-C	Proposed Method: Standard Increment: Blank D-C
125	0.186	0.022	0.200	0.005	0.199	0.009	0.201	0.011
	0.190	0.016	0.201	0.005	0.192	0.005	0.197	0.008
250	0.190	0.019	0.198	0.005	0.204	0.011	0.196	0.004
	0.189	0.020	0.196	0.003	0.200	0.009	0.198	0.007
500	0.187	0.020	0.205	0.005	0.200	0.012	0.199	0.009
	0.191	0.013	0.206	0.008	0.206	0.006	0.208	0.006

TABLE III.—PER CENT RECOVERIES OF FOLIC ACID IN PRESENCE OF VARIOUS RATIOS OF FERRIC AMMONIUM CITRATE ALONE AND WITH ASCORBIC ACID

Ratio	Method of Schiaffino, *et al.* — No Isobutyl — A[a]	No Isobutyl — B[b]	Isobutyl — A	Isobutyl — B	EDTA — A	EDTA — B	Proposed Method
200	97.6	100.0	96.0	98.2	93.7	99.4	101.7
200	96.6	101.0	100.2	103.1	98.9	103.6	101.3
400	89.9	N/D[c]	99.1	99.1	...	...	99.4
400	83.0	90.7	96.7	100.0	98.5	101.7	100.6
	65.1	65.7	100.0	105.7	97.1	100.0	...
800	67.8	75.0	81.7	81.7	97.0	99.0	101.8
800	20.9	N/D	266.6	266.6	99.4	102.4	98.2
400[d]	49.3	49.3	82.6	82.6	96.1	101.2	98.9
	94.2	100.3	100.5	108.9	97.8	101.8	101.9

[a] Uncorrected for standard blank. [b] Corrected for standard blank. [c] Not determinable. [d] With ascorbic acid at a ratio 150 to 1 of folic acid.

TABLE IV.—RECOVERY OF ADDED FOLIC ACID FROM LIVER EXTRACTS AND CONCENTRATES

Preparation	Added	Folic Acid Recovered, % — Method of Schiaffino, *et al.* — A[a]	Method of Schiaffino, *et al.* — B[b]	Proposed Method — Aqueous	Proposed Method — Isobutyl
Liver extract, injectable	5 mg./ml.	98.5	100.8	100.3	...
Liver concentrate 1:20 N. F.	1 mg./400 mg.	87.4	90.6	101.3	98.4
Elixir (2 Gm. liver ext./ml.)	0.5 mg./16 ml.	91.6	92.9	100.1	...
Elixir (rice bran + yeast ext.)	0.5 mg./16 ml.	70.5	71.4	100.0	98.4

[a] Uncorrected for standard blank. [b] Corrected for standard blank.

Determination of Folic Acid in Presence of Liver and Yeast Concentrates.—Folic acid was added to preparations containing liver extracts and concentrates and duplicate assays were made. Table IV gives the composition of these preparations and the results expressed in per cent of added folic acid. It can be seen that Schiaffino's method, even with the use of standard blanks and isobutyl alcohol extraction, gave complete recovery of folic acid only with the liver extract injectable. The modified method which included the use of extraneous color blanks gave complete recoveries in all tests. Results with isobutyl alcohol extraction for two of the preparations were only slightly lower. The preparations contained no appreciable amount of naturally occurring folic acid.

The lower results obtained by Schiaffino's method were found to be due to the absence of extraneous color blanks because when these were used with the K_2HPO_4 extracts from liver concentrate, results were similar to the proposed method.

Recoveries of Folic Acid from Multivitamin-Mineral Preparations.—To test the general applicability of the methods, folic acid was added to several different vitamin preparations, with or without various minerals and other ingredients. Each capsule or powdered tablet, free from any red dye, was added to the extracting solution followed by 0.5 mg. U. S. P. folic acid. Both procedures were used to assay for folic acid. Results obtained on six products which presented difficulties are shown in Table V. Recoveries in these products were markedly lower by the Schiaffino method, even when corrected by the use of a standard blank. They could not be attributed to any one constituent or group of factors. On the other hand, recoveries were essentially 100% by the proposed method.

More detailed studies were carried out on products A and B in an attempt to explain the difference observed between the results by the two methods.

TABLE V.—PER CENT RECOVERIES[a] OF FOLIC ACID IN PRESENCE OF CERTAIN FORMULATIONS

	Formulation	Schiaffino Method Without Std. Blank	Schiaffino Method With Std. Blank	Proposed Method
A	Multivitamin-mineral capsules (ferrous gluconate)	80.6	86.1	99.8
B	Multivitamin capsules	88.2	90.5	101.2
C	Multivitamin-mineral capsules (ferrous gluconate)	87.5	94.3	101.4
D	Multivitamin-mineral tablets (ferrous sulfate)	71.8	76.8	97.1
E	Multivitamin-mineral tablets (ferrous fumarate)	88.3	92.2	96.5
F	Multivitamin capsules (liver and ferrous sulfate, no ascorbic acid)	89.9	93.7	97.4

[a] Each value is the mean of 2 or 3 separate assays.

Folic acid was added either before or after extraction of the multivitamin samples with the appropriate extractant. With the original method, recovery was low when folic acid was added before extracting the sample, but higher when simply added after. Full recoveries were obtained with the proposed method when folic acid was added either before or after extraction.

Effect of PAB.—Because of the occasional presence of PAB in vitamin formulations, the effect of this substance on the estimation of folic acid was studied. Representative mixtures of equal weights of PAB

and folic acid either alone or in combination with two different multivitamin-mineral formulas were assayed in duplicate. As shown in Table VI, Schiaffino's method resulted in very low and erratic recoveries, while the proposed method, after removal of PAB with ethyl acetate containing benzoic acid, gave complete recoveries.

Table VI.—Per Cent Recoveries of Folic Acid in Presence of Equal Weights of PAB

Preparation	Method of Schiaffino, *et al.*	Proposed Method, Ethyl Acetate
PAB only	3.2	100.0
Multivitamin-mineral capsules (with ferrous gluconate and PAB)	38.1	100.0
Multivitamin-mineral capsules (with liver, ferrous sulfate, and PAB)	38.5	100.5

Other Interfering Materials.—Some reddish dyes, when present in the assay solution, interfered with the determination since they were decolorized in the tubes A and B ($KMnO_4$ oxidation) but remained in the blank tubes (without $KMnO_4$ oxidation). Isobutyl alcohol as recommended by Schiaffino, *et al.*, to extract the product of reaction also extracted a large quantity of the extraneous color. Consequently, the assay values for folic acid were decreased depending on the intensity of the extraneous color. When extraneous color blanks were used, higher assay values and better recoveries were found.

Hypophosphites and tryptophan were found to interfere with the permanganate oxidation determination of folic acid. Since folic acid is seldom, if ever, present in preparations containing hypophosphites or tryptophan no attempt was made to overcome the effect.

DISCUSSION

While the permanganate oxidation procedure described by Schiaffino, *et al.*, was found to have several advantages over the zinc reduction method, some difficulty was encountered with the recovery of folic acid in the presence of certain iron salts and constituents of common multivitamin-mineral preparations. Ferrous sulfate in the absence of ascorbic acid caused low recoveries of folic acid in this method. There was evidence from the blank readings that some splitting of folic acid occurred. The addition of ascorbic acid in the proportion of 150:1 of folic acid resulted in good recoveries of folic acid, thus overcoming the effect of ferrous sulfate. No explanation can be given for the finding of Schiaffino, *et al.*, that ferrous sulfate did not interfere under these conditions.

No difficulty was experienced with ferrous gluconate but ferric ammonium citrate produced a greenish interfering color in the Schiaffino method. This color may be removed with isobutyl alcohol extraction. The use of EDTA completely removed the interference of these substances and eliminated the need for isobutyl alcohol extraction. In the proposed method EDTA was necessary to remove interference caused by ferrous gluconate.

It was noted that with some reddish dyes and dark colored extracts, tubes A and B were decolorized with potassium permanganate but that the blank tube C was not. It was found that isobutyl alcohol extraction only partially removed these colors and that it was necessary to use special color blanks as described in the method.

In all of the comparisons made, the use of a standard blank in the original method resulted in better recoveries of folic acid. In the presence of multivitamin mixtures, the difference amounted to about 5%. It was apparently caused either by the reaction of a small proportion of the standard in the blank tubes and/or by the presence of small amounts of free amines in the standard. It was demonstrated that results obtained without employing a standard blank tube do not represent a true estimate of potency and therefore are not actually valid.

Difficulty was experienced in obtaining ready dispersion of the contents of certain capsules with 3% potassium phosphate. While the use of ethylene dichloride was helpful, it was found that the phosphate-lactate extractant which is proposed overcame this difficulty and offered several other advantages. In the presence of certain multivitamin-mineral preparations, full recovery of folic acid was obtained using this extractant while considerably less was obtained with the potassium phosphate extractant. An investigation of possible causes of this difference indicated that folic acid was not only more readily extracted from other factors by the combined extractant but also was more stable in it. The presence of a suspension in the extracting solution may seem unusual but much of the effectiveness of the procedure appears to depend on the excess of calcium lactate which protects folic acid in the extraction-precipitation process.

The permanganate oxidation procedure was found inapplicable in the few cases where PAB was present because the latter gave less absorbance in the tubes treated with potassium permanganate than in the blanks. To overcome this difficulty PAB was extracted with benzoic acid in ethyl acetate. Order of addition of reagents was modified to obtain the optimum pH of 4–6 in extraction. To compensate for possible loss of folic acid an internal standard was used prior to ethyl acetate extraction.

It may be concluded that the proposed method is applicable to the estimation of folic acid with a high degree of precision in the presence of most of the common constituents of multivitamin-mineral mixtures.

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Evaluation of an Oral Sustained Release Dosage Form of Trimeprazine as Measured by Urinary Excretion

By K. R. HEIMLICH, D. R. MacDONNELL, ANDREW POLK, and T. L. FLANAGAN

Effectiveness of sustained release dosage forms of trimeprazine tartrate, as pellets with varying thicknesses of a slowly digestible wax-fat mixture, is measured by determining the urinary excretion of the drug or its metabolites during specified periods after ingestion. Results are compared with *in vitro* tests.

ONE OF THE GREATEST difficulties encountered in the development of sustained release dosage forms is the procurement of human *in vivo* data which adequately demonstrate the sustained release properties of the dosage form. Although clinical efficacy is usually the foremost test in determining if the preparation is satisfactory, a concerted effort should be made to obtain quantitative *in vivo* data as a guide in selecting the most suitable formula.

Once the desired formula has been selected, then *in vitro* release rate specifications can be finally established by various methods to insure the reproducibility of subsequent batches.

Recent studies reported in the literature (1–9) have considered many of the important theoretical aspects of sustained release formulations. Some of these studies have shown how a knowledge of blood level, urinary excretion, and biological half-life data, when related to therapeutic response, may be used to design and evaluate a sustained release form. The application of these principles in the development of sustained release forms of many drugs is not always feasible.

The biological half-life as obtained from blood or urine data may not have a direct relationship to the therapeutic response in question. In these cases it may be necessary to obtain the biological half-life from some physiological response or side effect which can be objectively measured, even though the response may not be the specific action for which the drug is intended. Also, in order to apply the mathematical expressions which may be used to interpret urinary excretion data, a period of constant rate of excretion must be demonstrated after administration of the sustained release form. During the short time normally used in conducting an excretion study, this so-called "steady state" period may not be evidenced by some drugs which undergo complicated excretion-absorption cycles or involved detoxification, and other methods of evaluating the data must be used.

It is possible to design and evaluate sustained release formulations by comparing urinary excretion data obtained after the administration of unprocessed drug three or four times a day with the excretion data when the sustained release form is given in an equal, unit dose. This regimen is based on the fact that, in most instances, the primary objective of sustained release medication is to replace existing single-dose forms usually given every three or four hours. If the sustained release formulation is not suitable, the data will be useful in making the revisions necessary to obtain an acceptable product.

The present study was carried out to test the suitability of a sustained release form of trimeprazine tartrate.[1] The specific objectives of the study were: (*a*) to compare the physiological availability and urinary excretion patterns of trimeprazine or its metabolites in subjects administered the sustained release form or an equivalent amount of unprocessed drug given in three divided doses, (*b*) to determine the degree of completeness of disintegration in humans of material having an *in vitro* release of at least 80 per cent in seven hours by examination of stool specimens for undisintegrated pellets, and (*c*) to compare the results of tests (*a*) and (*b*) with data obtained when a sustained release form having an *in vitro* release of approximately 50 per cent in seven hours was administered to humans.

EXPERIMENTAL

Preparation of the Dosage Form.—The sustained release dosage forms were prepared following the general procedure given by MacDonnell (10). A trimeprazine tartrate, starch, and powdered sugar coating powder was applied to small cores using a

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[1] The trade name of Smith Kline and French for this product is Temaril Spansule.

gelatin adhesive solution. Successive coatings of the drug dilution were applied until the proper concentration of drug was obtained. The sustained release properties were effected by coating sub-batches of medicated pellets with varying thicknesses of a slowly digestible wax-fat material using Tecsolv No. 388[3] as a solvent for the coating material. Mixes having varying rates of release were then obtained by blending uncoated medicated pellets with various sub-batches of wax-fat coated pellets.

The *in vitro* releases of the dosage forms were determined using the general method given by Souder and Ellenbogen (11) and are listed in Table I.

TABLE I.—*In Vitro* RELEASES OF SUSTAINED RELEASE FORMS

Simulated Gastric Fluid T. S., U. S. P. XV, hr.		Simulated Intestinal Fluid T. S., U. S. P. XV, hr.	Per Cent of Drug Released —per Hourly Interval— Formula F	Formula H
1/2			32	1
1 1/2	+	1/2	39	6
1 1/2	+	3	76	29
1 1/2	+	5 1/2	93	47

Dosage Regimen.—The study was carried out in a series of three tests. In test No. 1, each of three subjects received 30 mg. of trimeprazine in sustained release form (formula F), and three subjects received 30 mg. of unprocessed trimeprazine administered in 10-mg. doses every four hours. Test 2 was a crossover of test 1. In test 3, all six subjects received 30 mg. of trimeprazine in a sustained release form (formula H) which had a seven hour *in vitro* release of approximately 50%.

Specimen Collection.—A control urine specimen was collected immediately prior to the ingestion of the dosage forms. All urine voided during the following time periods was collected: zero to four, four to eight, eight to twelve, twelve to twenty-four, and twenty-four to thirty-six hours. When sustained release forms were administered, stool specimens were collected by each subject for a period of forty-eight hours or until two specimens were obtained.

Analysis of Urine Specimens.—The analytical procedure used for the determination of trimeprazine in the urine specimens is an adaptation of Eiduson's (12) procedure. This is a resin extraction method first reported by Berti and Cima (13) for chlorpromazine in biological fluids and later adapted to analysis of urines by Eiduson. The method is not specific for unchanged trimeprazine since any metabolites, bearing the phenothiazine nucleus, adsorbed on the resin are determined colorimetrically as trimeprazine. Also preliminary studies in these laboratories with trimeprazine-S^{35} indicated that the resin method does not extract conjugated derivatives which may be formed during the metabolism of the compound. It was not felt necessary to determine the amount and nature of the metabolites of the trimeprazine excreted since the evaluation was a crossover study comparing unprocessed drug and sustained release dosage forms.

[3] Tect and Co.

Stool Specimen Analysis.—The stool specimens were examined by a qualified parasitologist for any undisintegrated pellets. This was done by gently slurrying the specimens in water and screening the slurry through a series of sieves (12–16–35 mesh). By this technique any pellets present in fecal material were recovered and counted.

RESULTS

Table II presents the results of the determinations on urine specimens.

The examination of stool specimens revealed more shells and fragments of pellets after the administration of the formula H material than after the administration of formula F. However, analysis of the material recovered from both formulations showed insignificant amounts of trimeprazine to be present. This could be expected since the total availability of both sustained release forms was the same as the plain drug.

DISCUSSION OF RESULTS

Criteria which may be used to judge the adequacy of a sustained release formulation are as follows: (*a*) The urine levels in the first collection period after administration of the unprocessed drug and the sustained release form should be nearly equivalent. This will indicate that the sustained release form is releasing sufficient medication initially. (*b*) The urine levels in the later collection periods or for the period for which sustained action is claimed should be equivalent from the two dosage forms to indicate adequate duration of effect from the sustained release formulation. (*c*) The total availability or recovery from the sustained release form should be equivalent to that of the unprocessed drug. (*d*) Examination of stool specimens from subjects receiving the sustained release form should not reveal undisintegrated pellets.

To check the formulations against the first two criteria, the average amounts of trimeprazine or metabolites excreted per period from each formulation were compared statistically. The amounts excreted after the administration of the unprocessed drug and the formula F sustained release material compared closely for the period of therapeutic interest. There was no significant difference in the amounts excreted for the zero to four-hour period or the eight to twelve-hour period as determined by a *t* test on the means (95% confidence level). The excretion for the four to eight-hour period was somewhat higher after administration of the formula F material. These results indicate that a sufficient amount of drug was released initially from the sustained release form and that the release continued at a rate which supplied sufficient drug for absorption to maintain an excretion level equivalent to that after the unprocessed drug dosage for a twelve-hour period.

The amounts of trimeprazine or metabolites excreted during the first three collection periods after the administration of the formula H sustained release material appeared to be consistently low. Although these differences from the unprocessed drug were not significant at the 95% confidence level, calculation indicated that the use of three or four more subjects would have shown these dif-

TABLE II.—TRIMEPRAZINE OR METABOLITES PRESENT IN URINE SAMPLES AT VARIOUS TIME INTERVALS

Subject[a]	Dosage Form	Mg. of Trimeprazine or Metabolites Per Collection Period, hr.— 0–4	4–8	8–12	12–24	24–36	0–36	Per Cent Recovery
V. C.	Unprocessed drug, 30 mg. (10 mg. every 4 hours)	0.5	1.9	2.4	3.3	3.0	11.1	37.0
Mrs. V. C.		0	0.7	1.8	5.0	1.8	9.3	31.0
C. L.		0.4	0.8	1.9	3.6	0	6.7	22.3
A. M. J.		0.3	0.4	1.4	2.1	0.9	5.1	17.0
C. N.		1.8	1.4	3.7	0.6	0	7.5	25.0
Average		0.6	1.0	2.2	2.9	1.1	7.9	26.5
V. C.	Sustained release formula F (six 5-mg. units) (30 mg.)	1.0	2.1	3.4	1.0	1.5	9.0	30.0
Mrs. V. C.		0.7	1.3	2.1	3.0	2.6	9.7	32.3
C. L.		1.0	2.5	2.6	6.5	1.5	14.0	47.0
A. M. J.		0.8	0.9	1.7	4.3	2.3	10.0	33.0
C. N.		0.6	1.8	2.3	9.2	5.4	19.3	64.3
Average		0.8	1.7	2.4	4.8	2.7	12.4	41.3
V. C.	Sustained release formula H (six 5-mg. units) (30 mg.)	0.2	1.5	2.1	2.0	3.5	9.3	31.0
Mrs. V. C.[b]		0	0	0	3.2	2.4	5.6	22.4
C. L.		0	0.8	1.5	3.4	1.7	7.4	24.7
A. M. J.		0.2	0.3	1.0	2.2	1.4	5.1	17.0
C. N.		0.8	0.6	2.7	4.4	2.3	10.8	36.0
Average		0.2	0.6	1.5	3.0	2.3	7.6	26.2

[a] One subject withdrew from the study. [b] Total dose was 25 mg. of trimeprazine.

ferences to be significant. Thus the formula F but not the formula H material fulfilled the first two criteria.

From a cursory examination of the data, it appears as though there might be an increased total excretion of trimeprazine or its metabolites after the administration of formula F. However, from past experience with sustained release dosage forms, it would be expected that the recovery be no more than equivalent to the recovery after administration of the nonsustained release form. This was found to be the case by a *t* test on the mean total recoveries. There are instances in the literature in which total urinary excretion of drug has been greater after the administration of a special dosage form as compared to unprocessed drug administration (14, 15). Further urinary excretion studies would be required to permit any other conclusion than an equivalency of recovery with the present dosage form.

Since no appreciable amounts of drug were found in pellet fragments recovered in fecal specimens, formula F fulfilled all four criteria and was accepted as a satisfactory formulation. The formula H material, although physiologically available over the total test period, did not show adequate *in vivo* release during the early periods and was not considered acceptable.

CONCLUSIONS

1. The urinary excretion of trimeprazine or its metabolites for a twelve-hour period following the administration of 30 mg. of trimeprazine in the form of six 5-mg. sustained release capsules (formula F) closely duplicated the excretion obtained upon the administration of an equal amount of unprocessed drug in three divided doses every four hours. The sustained release form was also shown to be physiologically available as evidenced by the comparative total recoveries.

2. A sustained release form of trimeprazine (formula H) having a significantly slower *in vitro* release rate was physiologically available but was judged unsatisfactory because of insufficient amounts of drug or metabolites excreted in the early collection periods as compared to the unprocessed drug.

3. The statistical comparison of urinary recovery after the administration of sustained release dosage forms and after divided dose medication of unprocessed drug is a useful and convenient method of evaluating sustained release dosage forms.

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Experimental Investigation of Nine Antitussive Drugs

By P. L. STEFKO, J. DENZEL, and I. HICKEY

A series of chemically unrelated compounds were evaluated and compared as to their relative cough suppressant activity in a group of varying test subjects. Graded responses were noted and detailed in the report.

A NUMBER of chemically unrelated preparations have been reported to have cough suppressant activity. The hydrobromides of the dextro, levo, and racemic forms of 3-methoxy-N-methylmorphinan were reported by Benson, Randall, and Stefko (1) to have antitussive activity in dogs. Cough suppression was more marked with the dextro-3-methoxy-N-methylmorphinan hydrobromide (dextromethorphan hydrobromide)[1] than with the racemic form; the dextro isomer did not produce central depression. Isbell and Fraser (2) who studied the hydrobromides of the optical isomers of 3-methoxy-N-methylmorphinan in former morphine addicts concluded that dextromethorphan hydrobromide is devoid of addiction potentialities. Cass and Frederick (3) concluded that for all practical purposes dextromethorphan hydrobromide and codeine may be considered of equal antitussive effectiveness on a weight basis in their "double blind" study on sixty-three subjects with chronic cough.

Levis, Preat, and Moyersoons (4) studied carbetapentane citrate [2-(2-diethylaminoethoxy)ethyl 1-phenylcyclopentanecarboxylate citrate] and found it was highly effective in suppressing the cough in an anesthetized cat. Analysis of their data showed that carbetapentane citrate was more effective than codeine phosphate. Parish (5) reported on the clinical effectiveness of carbetapentane citrate. Relief was obtained with carbetapentane citrate in minutes and maintained for hours in cough associated with acute coryza.

Bucher (6, 7, 8) used the technique described by Kroepfli (19) of inducing a cough in anesthetized cats by irritating the trachea with insufflation of powdered soap to demonstrate the cough suppressant activity of benzonatate (nonaethyleneglycol monomethyl ether *p*-*n*-butylaminobenzoate). The investigations showed that benzonatate is a nonnarcotic and pharmacologically acts both peripherally and centrally on the cough reflex. Herzay (9) has shown clinically that benzonatate has a cough-relieving effect, exerting a central action but does not have the typical effects of morphine and its derivatives.

Caramiphen ethanedisulfonate (1-phenyl-1-cyclopentanecarboxylic acid *β*-diethylaminoethyl ester ethanesulfonate) was reported by Toner and Macho (10) and by Chakravartz, Mattallana, Jensen, and Borison (11) to have marked cough suppressant action in their anesthetized cat preparations. On the basis of intensity and duration, 5 mg./Kg. of caramiphen ethanedisulfonate was about as effective as 1 mg./Kg. of codeine phosphate. The drugs were administered intravenously. Ablemann, Gaensler, and Badger (12) demonstrated clinically that caramiphen ethanedisulfonate given orally was accompanied by partial to considerable relief from distressing cough in 57 per cent of patients.

Rosiere, Winder, and Wax (13) in a comparative antitussive bioassay of four morphine derivatives and methadone demonstrated that dihydrocodeinone bitartrate was seven to eight times as potent as codeine phosphate. Ammonia cough elicited through a tracheal side tube in anesthetized dogs was utilized in this study. Rudner (14) evaluated dihydrocodeinone bitartrate clinically in tuberculosis cough and reported the new codeine derivative to be more effective than codeine and to possess a low incidence of side reactions.

One of the most widely employed tests for the study of antitussive activity is the Winter-Flataker sulfuric acid spray technique. This test was used by Chen, Biller, and Montgomery (15) to evaluate the efficacy of a new nonnarcotic antitussive drug, *α*-(dimethylaminoethyl)-*ortho*-chlorbenzhydrol hydrochloride. The authors compared this compound under standard experimental conditions with codeine phosphate, dicodid, noscapine, dextromethorphan, dimethoxanate, and benzonatate. It was found to possess a degree of antitussive potency comparable to that of dicodid, with a relatively latent onset of maximum action but a prolonged duration of action.

The purpose of this report is to survey recent developments in the field of antitussive drugs, with the intention of clarifying cough suppressant

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[1] Romilar is the Hoffmann-La Roche trademark for dextromethorphan.

activity in animals. A review of the literature revealed that, while there is a plethora of new methods and devices worthy of comment, none involves principles hitherto undescribed.

The evaluation of the drugs was carried out by two methods developed by the senior author (16, 17). There are a number of methods described in the literature (18–29) of quality rating. Some involve direct stimulation of the cough center (16). For an estimation of relative potencies of drugs, it is necessary to apply quantitative methods.

In these experiments, the nine antitussive drugs were tested on the cough resulting from chemical irritation and cough elicited by electrical stimulation of the tracheal submucosa. The results illustrate that the drugs evaluated significantly inhibited the cough in the cat and dog under our experimental conditions.

METHODS AND MATERIALS

Unanesthetized Dog Preparation.—In the unanesthetized dog, antitussive activity was determined by the electrical stimulation of the trachea through previously implanted copper electrodes into the tracheal submucosa (30). The evaluation of antitussive activity is based on a numerical rating system and scored as per cent inhibition. The individual responses of each animal to a drug is graded by comparing the number and severity of the cough before and after drug therapy.

The preparations were given orally. The antitussive activity was studied over a period of two hours. Careful observations were made of any concomitant effects.

Anesthetized Cat Preparation.—In an anesthetized cat, cough attacks were provoked by the sudden introduction of ammonia into the airways. This was accomplished by a snugly fitting mask about the animal's snout. The ammonia was introduced at the end of expiration and the cough in all instances occurred at the next inspiration. The respiration, amplitude, and duration of the response were recorded on a smoked drum using a pneumograph and tambour.

A control period was obtained by subjecting the animal to the ammonia fumes once every three minutes for twelve minutes; four control cough responses of equal intensity and duration were obtained. The changes in the cough attacks following drug administration were recorded every five minutes until a return to the normal control response. The ammonia concentration found suitable was 7% (as NH_3) (31).

Pathologic Cough in Dogs.—The drugs evaluated in this study were also given to dogs with cough resulting from an upper respiratory infection.

All compounds were studied and administered as crystalline salts. For purpose of simplicity, future reference to these agents will be made by generic name without designation to trade names (except in the case of α-(dimethylaminoethyl)-*o*-chlorbenzhydrol hydrochloride which will be referred to as B-186). The chemical and generic names of the drugs used in these studies appear below.

Chemical Name	Generic Name
Codeine phosphate	.....
Morphine sulfate	.....
Dihydrocodeinone bitartrate	hydrocodone bitartrate
dextro-3-Methoxy-N-methylmorphinan hydrobromide	dextromethorphan hydrobromide
Nonaethyleneglycol monomethyl ether *p*-*n*-butylaminobenzoate	benzonatate
1-Phenyl-1-cyclopentanecarboxylic acid β-diethylaminoethyl ester	caramiphen ethanedisulfonate
2-(2-Diethylaminoethoxy)ethyl 1-phenylcyclopentanecarboxylate citrate	carbetapentane citrate
N-Methyl-2,4-methylenedioxyphenethylamine hydrochloride	homarylamine hydrochloride
α-(Dimethylaminoethyl)-*ortho*-chlorbenzhydrol hydrochloride	.....

RESULTS AND DISCUSSION

Table I illustrates the effect of oral administration of various doses of morphine sulfate, codeine, dextromethorphan, carbetapentane citrate, benzonatate, caramiphen ethanedisulfonate, hydrocodone, homarylamine hydrochloride, and B-186 on the cough response due to an electrical stimulation of

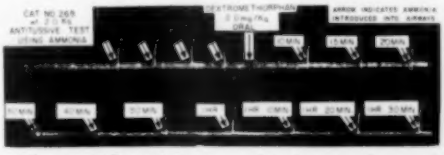

Fig. 1.—Effect of dextromethorphan on the cough attacks produced by the inhalation of ammonia into the airways of an anesthetized cat.

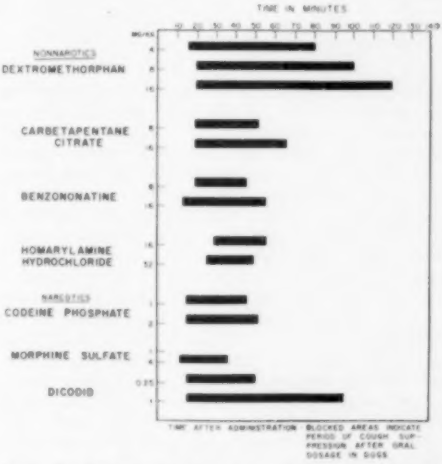

Fig. 2.—Effect of cough supressants, narcotic and nonnarcotic, in pathologic cough in dogs.

TABLE I.—COUGH SUPPRESSANT ACTION OF A NUMBER OF DRUGS IN THE UNANESTHETIZED DOG PREPARATION

Drug	Dose, mg./Kg. P. O.	No. of Dogs	No. of Expts.	Average Per Cent Inhibition of Number of Coughs, min. 15	30	60	120
Codeine phosphate	0.25	10	15	9	18	24	10
	0.5			14	22	30	12
	1.0	12	14	26	46	44	30
	2.0	9	15	44	78	76	52
	4.0	11	16	48	88	99	78
Morphine sulfate	0.25	9	11	0	12	16	0
	0.5	12	14	10	20	20	15
	1.0	12	16	12	28	32	14
	2.0	10	14	32	48[a]	42[a]	30[a]
	4.0	12	16	22[a]	52[b]	49[b]	34[b]
Dextromethorphan	0.5	12	27	16	28	23	12
	1.0	12	28	30	46	54	32
	2.0	15	30	42	63	68	44
	4.0	13	28	44	76	84	54
	8.0	11	24	48	84	90	70
	16.0	10	12	48	90	92	70
Benzonatate	0.5	4	4	0	17	38	11
	1.0	4	4	0	33	82	14
	4.0	4	4	0	61	90	40
	8.0	4	4	0	75	90	46
	16.0	4	4	0	90	100	58
Carbetapentane citrate	0.5	4	6	0	20	31	11
	1.0	4	6	0	44	65	30
	2.0	4	6	0	43	68	28
	4.0	3	5	14	47	80	40
	8.0	3	5	0	46	81	42
	16.0	3	5	22	81	100	40
Caramiphen ethanedisulfonate	4.0	5	5	0	1	0	1
	8.0	4	4	0	4	4	4
	16.0	5	5	0	2	0	0
	32.0	3	4	0	0	0	0
Dihydrocodeinone bitartrate	0.1	4	4	1	23	45	11
	0.25	3	4	10	40	73	44
	0.5	4	4	13	67	93	73
	1.0	4	4	20	100[a]	100[b]	84[b]
	2.0	4	4	40[a]	100[b]	100[b]	82[b]
Homarylamine hydrochloride	0.5	4	4	0	2	11	0
	2.0	3	4	10	28	38	12
	8.0	4	6	12	40	42	18
	16.0	4	6	18	67	70	28

Time, min.		No. of Expts.	Time, min. 15	30	60	90	120	150	180	210	240	270
B-186	1.0	2	0	0	0	0	17	17	3	0	0	0
	2.0	2	0	0	3	17	40	33	17	0	0	0
	4.0	3	0	0	17	40	50	50	40	17	0	0
	8.0	2	0	0	33	50	66	66	50	33	20	0

[a] Marked sedation. [b] Animal asleep.

the tracheal submucosa in the unanesthetized dog preparation. It can be seen that dextromethorphan compares quite favorably with the other antitussive preparations. There is a good relationship of dose and effect. It can be seen that at all dose levels, dextromethorphan is just as effective as codeine, carbetapentane citrate, and benzonatate, and more effective than caramiphen ethanedisulfonate, homarylamine hydrochloride, and morphine sulfate, but less effective than hydrocodone. The antitussive effects of dextromethorphan differ from codeine, carbetapentane citrate, benzonatate, homarylamine hydrochloride, and B-186 in that these preparations need a period of incubation for their full activity to develop. Dextromethorphan has a rapid onset of action, achieving essentially full effectiveness within thirty minutes after oral administration. Benzonatate and carbetapentane citrate in particular require one-half hour for antitussive action to become measurably significant and do not reach peak effectiveness until one hour after administration; following this there is a very rapid decline in antitussive effect.

B-186 exhibited a marked degree of antitussive potency not comparable however to hydrocodone, codeine, carbetapentane citrate, and benzonatate. The drug had a relatively latent onset of maximum action but a prolonged duration of action.

No adverse central effects were observed with dextromethorphan. Hydrocodone in doses of 0.5 mg./Kg. and above causes undesirable side effects. The adverse effects consist of lethargy, sleep, and defecation.

Caramiphen ethanedisulfonate, although a very powerful antitussive preparation when administered intravenously, is inactive when administered orally in the unanesthetized dog preparation.

Figure 1 illustrates the effect of dextromethorphan on the cough attacks produced by the inhalation of ammonia into the airways of an anesthetized cat.

The antitussive activity of varying doses of dextromethorphan, codeine, morphine sulfate, ben-

TABLE II.—ANTITUSSIVE EFFECTIVENESS OF A NUMBER OF DRUGS IN THE ANESTHETIZED CAT PREPARATION

Drug	Dose, mg./Kg. P. O.	No. of Cats	No. of Expts.	Activity	Duration of Action, min.	Remarks
Codeine phosphate	0.25	8	9	Active	15	...
	0.5	12	12	Mod. active	31	...
	1.0	10	12	Markedly active	38	...
	2.0	12	12	Markedly active	50	...
	4.0	12	12	Markedly active	78	...
Morphine sulfate	0.25	4	6	None	...	...
	0.5	4	6	Slightly active	6	...
	1.0	6	6	Slightly active	10	...
	2.0	6	6	Slightly active	14	...
	4.0	5	6	Active	20	...
	8.0	6	6	Active	28	Respiratory depression
Dextromethorphan	0.25	10	10	None	...	...
	0.5	12	12	None	...	...
	1.0	12	12	Active	20	...
	2.0	12	12	Active	30	...
	4.0	12	12	Mod. active	42	...
	8.0	12	12	Markedly active	50	...
	16.0	12	12	Markedly active	68	...
Benzonatate	0.1	6	6	None	...	...
	0.25	8	8	None	...	...
	0.5	6	6	None	...	...
	1.0	6	6	Slightly active	5	...
	2.0	8	8	Slightly active	10	...
	4.0	6	6	Mod. active	30	...
	8.0	8	8	Mod. active	42	...
	16.0	10	10	Markedly active	72	...
Carbetapentane citrate	1.0	6	6	None	...	...
	2.0	6	6	Slightly active	10	...
	4.0	8	8	Mod. active	25	...
	8.0	8	8	Mod. active	35	...
	16.0	8	8	Markedly active	60	Respiratory depression
Caramiphen ethanedisulfonate[a]	8.0	6	6	None	...	...
	16.0	6	6	None	...	...
	32.0	6	6	None	...	...
Dihydrocodeinone bitartrate	0.1	6	6	Mod. active	30	...
	0.25	6	6	Markedly active	55	...
	0.5	6	6	Markedly active	68	Respiratory depression
	1.0	6	6	Markedly active	80	...
Homarylamine hydrochloride	4.0	5	5	None	...	...
	8.0	6	6	Slightly active	8	...
	16.0	6	6	Mod. active	22	...
	32.0	6	6	Mod. active	30	Respiratory stimulation
B-186	1.0	1	1	None	...	...
	8.0	3	3	Mod. active	90	...
	16.0	3	3	Mod. active	125	...

[a] Caramiphen ethanedisulfonate is markedly active in intravenous doses of 0.25 to 4 mg./Kg.

zonatate, carbetapentane citrate, homarylamine hydrochloride, and hydrocodone in the anesthetized cat preparation are summarized in Table II. The data reveal that hydrocodone is most active in suppressing the cough attacks induced by a chemical irritant, ammonia, in an anesthetized cat preparation. Next in order of cough suppressant activity are codeine, dextromethorphan, benzonatate, carbetapentane citrate, homarylamine hydrochloride, and morphine sulfate. Caramiphen ethanedisulfonate is inactive when administered orally.

The activities of 2 mg./Kg. dextromethorphan, benzonatate, and carbetapentane citrate are equivalent to 0.1 mg./Kg. hydrocodone. Thus, the latter may be considered 20 times as active as the former drugs. The duration of action of 2 mg./Kg. dextromethorphan and 0.1 mg./Kg. hydrocodone is thirty minutes. Dextromethorphan is more active than carbetapentane citrate, benzonatate, and homarylamine hydrochloride. Caramiphen ethanedisulfonate is inactive.

The comparative results of the effect of oral administration of various doses of codeine, morphine sulfate, dextromethorphan, benzonatate, carbetapentane citrate, homarylamine hydrochloride, caramiphen ethanedisulfonate, and hydrocodone on the cough resulting from a respiratory infection in dogs are shown in Table III and are graphically presented in Fig. 2. These animals exhibited a postdistemper or postpneumonitis cough. It has been found that dicodid and dextromethorphan are very effective in inhibiting the "pathologic" cough. Hydrocodone is effective but caused undesirable side effects at the therapeutic dosage level. The side effects consist of lethargy, sleep, and defecation.

TABLE III.—COUGH SUPPRESSANTS IN PATHOLOGIC COUGH ORALLY IN DOGS

Dog No.	Dose, mg./Kg. P. O.	Wt., Kg.	Drug	Remarks
20	4	8.2	Dextromethorphan	Animal ceased coughing in 15 min.; duration of action, 65 min.
21	8	7.1	Dextromethorphan	Animal ceased coughing in 20 min.; duration of action, 80 min.
22	16	9.8	Dextromethorphan	Animal ceased coughing in 20 min.; duration of action, 100 min.
23	16	5.4	Caramiphen ethanedisulfonate	No effect
24	32	8.1	Caramiphen ethanedisulfonate	No effect
23	0.25	5.2	Dihydrocodeinone bitartrate	Animal ceased coughing in 15 min.; duration of action, 35 min.
25	1.0	6.2	Dihydrocodeinone bitartrate	Animal ceased coughing in 15 min.; duration of action, 80 min; lethargy, sleep, 30 min.
24	8	8.0	Carbetapentane citrate	Cough diminished in 20 min.; duration of action, 30 min.
26	16	7.6	Carbetapentane citrate	Animal ceased coughing in 20 min.; duration of action, 45 min.
27	8	9.7	Benzonatate	Cough diminished but not inhibited; onset of action, 20 min.; duration of action, 25 min.
28	16	10.4	Benzonatate	Animal ceased coughing in 15 min.; duration of action, 40 min.
29	16	7.0	Homarylamine hydrochloride	Animal ceased coughing in 30 min.; duration of action, 20–25 min.
30	32	11.2	Homarylamine hydrochloride	Animal ceased coughing in 25 min.; duration of action, 25 min.
31	1.0	8.8	Codeine phosphate	Animal ceased coughing in 15 min.; duration of action, 30 min.
32	2.0	7.8	Codeine phosphate	Animal ceased coughing in 15 min.; duration of action, 38 min.
33	1.0	12.6	Morphine sulfate	No effect
34	4.0	9.2	Morphine sulfate	Animal ceased coughing in 15 min.; duration of action, 25 min.; lethargy def.

Codeine, benzonatate, carbetapentane citrate, morphine sulfate, and homarylamine hydrochloride are moderately active. Caramiphen ethanedisulfonate is inactive.

The results reported in this entire study have significance only for the specific conditions of testing. It is interesting that dextromethorphan compares favorably with the other nonnarcotic cough suppressants. Of significance is the activity of dextromethorphan demonstrated against "experimental" cough in the dog which was confirmed by the activity against "pathologic" cough in the same species. Only hydrocodone was found to be more active than dextromethorphan in this test. However, hydrocodone, at the effective dosage, caused undesirable side effects.

SUMMARY

Nine chemically unrelated preparations were evaluated for their cough suppressant activity in the unanesthetized dog, in the anesthetized cat preparations, and on the "pathologic" cough in dogs with an upper respiratory infection.

Dextromethorphan has a cough suppressant effect which is fairly rapid in onset. The potency and duration of action are comparable to codeine, benzonatate, and carbetapentane citrate. Hydrocodone possessed greater antitussive activity.

In the unanesthetized dog preparation oral doses of dextromethorphan, carbetapentane citrate, benzonatate, codeine, and hydrocodone markedly suppressed the cough response; homarylamine hydrochloride and morphine sulfate were moderately active, while caramiphen ethanedisulfonate was inactive. Hydrocodone exhibited side effects at the effective dosage levels.

Dextromethorphan, codeine, hydrocodone, carbetapentane citrate, benzonatate, and B-186 administered orally to the anesthetized cat preparation markedly suppressed the cough attacks; homarylamine hydrochloride and morphine sulfate moderately suppressed the cough attacks; caramiphen ethanedisulfonate was inactive.

Oral doses of dextromethorphan, codeine, and hydrocodone markedly suppressed the "pathologic" cough in the dog, due to an upper respiratory infection; benzonatate, carbetapentane citrate, morphine sulfate, and homarylamine hydrochloride moderately suppressed the cough; caramiphen ethanedisulfonate was inactive.

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Protection of Yeast Against Photosensitized Killing

By JOSEPH JUDIS

The ability of a number of radioprotective compounds to protect *Saccharomyces cerevisiae* against photosensitized killing was studied. Protection was afforded by cysteamine, glutathione, penicillamine, N,N-dimethylmercaptoethylamine, 1-mercapto-2-aminobutane, mercaptopropylamine, 2-aminoethylisothiouronium bromide HBr (AET), and cysteine, but not by cystamine, potassium cyanide, or glucose. Other sulfhydryl compounds such as mercaptosuccinic acid, β-mercaptopropionic acid, sodium thioglycollate and α-monothioglycerol also did not protect. It appears from the data presented that the thiol compounds with a free or substituted amino group protected yeast against photodynamic killing while the thiol compounds lacking the amino group did not protect.

THE TERM "photodynamic action" was first applied by Raab (1) to his observation of the lethal photosensitization of protozoa and this phenomenon has since been the subject of considerable literature (2–4). Photodynamic action is of interest not only for theoretical reasons but, on the practical level, a number of diseases of animals have been traced to photosensitization and several medicinal substances have been shown to cause photosensitization as a side reaction (2, 3, 5, 6). While photodynamic action has been reasonably well studied, its mechanism remains obscure. It appeared to the author that an approach to understanding the cause of the biological damage might be facilitated by a study of chemical substances that are capable of preventing this damage. Obviously, excited molecules of some sort are involved and because the possibility exists that these molecules might be free radicals, it seemed reasonable to examine the effect on photosensitized killing of yeast of a number of compounds known to protect against ionizing radiation, the mechanism of the biological action of which is believed to involve free radicals (8).

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MATERIALS AND METHODS

Materials.—The chemicals used were of C. P. grade. Biochemicals were obtained from the California Corporation for Biochemical Research, Nutritional Biochemicals Corporation, and Schwartz Laboratories. The β-mercaptopropionic acid and α-monothioglycerol were obtained from Evans Chemetics, Inc., and the 1-mercapto-2-aminobutane HCl, N,N-diethylmercaptoethylamine HCl, and mercaptopropylamine HCl were a gift from Dr. David Jacobus of the Walter Reed Army Medical Center.

Procedure.—The irradiation apparatus consisted of a bank of two Sylvania 150-watt projector spot lamps and the yeast suspensions were contained in 16- x 150-mm. screw cap, Kimax test tubes mounted on a Fisher Gyrosolver shaker to provide a uniform suspension during the treatment. The heat from the lights was dissipated by an electric fan and by

placing a Blake bottle, through which water was circulated, between the lights and the test tubes, resulting in a temperature of 28 ± 3° in the yeast suspensions. The distance between the bulbs and the test tubes was 20 cm., at which point the illumination was 3,100 foot candles as measured by a GE standard light meter. The usual reaction mixture contained, in a total of 5 ml., 150×10^6 yeast cells, 1.14×10^{-6} *M* concentration of erythrosin as the photosensitizer, and pH 7.2 phosphate buffer in a final concentration of 0.067 *M*. A stock, diploid, strain of *Saccharomyces cerevisiae* was used throughout and was cultured on slants of Sabouraud's maltose agar. After twenty-four hours of incubation at 25°, the growth from a slant was washed off with distilled water, washed once with water, and resuspended in an amount of 0.067 *M*, pH 7.2 phosphate buffer to give the desired concentration of cells. After irradiation, the suspensions were centrifuged, resuspended in distilled water, and viable yeast cells estimated by the standard pour plate procedure. All work was done in subdued light in order to control the dose of radiant energy applied. A number of controls were included: (*a*) yeast plus dye in the dark; (*b*) yeast minus dye in the light; (*c*) yeast minus dye in the dark. Where various substances were added to determine their protective powers, the former were incubated on the shaker with yeast in the light or with yeast plus dye in the dark as additional controls to determine possible toxic effects of the substances on the yeast unrelated to photodynamic action.

RESULTS AND DISCUSSION

A typical killing curve is given in Fig. 1. The various controls showed no significant change in viable count from untreated yeast suspensions. Killing occurred only when both light and dye were simultaneously present. This type of killing curve is similar to those reported for bacteria and viruses (7, 9, 10).

A number of compounds have been reported to inhibit the biological effects of photodynamic action, such as sodium sulfite and sodium thiosulfate (2, 9), proteins (11, 12), ascorbic acid (13), cysteine (9, 14), reduced glutathione (9), sodium sulfide (9), and agar (15). The mechanism of photodynamic action (2, 4, 7, 16–19) undoubtedly includes at least the following steps: (*a*) adsorption of the dye by the susceptible cell or combination with the substrate; (*b*) adsorption by the dye, in the above complex, of the light energy with the formation of an activated dye molecule; (*c*) combination of the activated dye-substrate complex with oxygen to form an oxidizing substance; (*d*) oxidation of the substrate with return of the dye to the ground state, thus available to repeat the cycle. A protective agent could presumably act by inhibiting any of the above steps. Strong reducing agents, such as the inorganic sulfur compounds, could act by making oxygen unavailable or altering the dye chemically so that it would not be able to function in the photochemical reaction. Probably the site of biological damage would be the —SH group which is subject to oxidation, although a number of amino acids have been shown to be destroyed by photodynamic action, such as histidine and tryptophan (4, 20) possibly in the cell wall (7). The photodynamic inactivation of viruses would tend to indicate the possibility that nucleoproteins may be a site of attack also (21, 22).

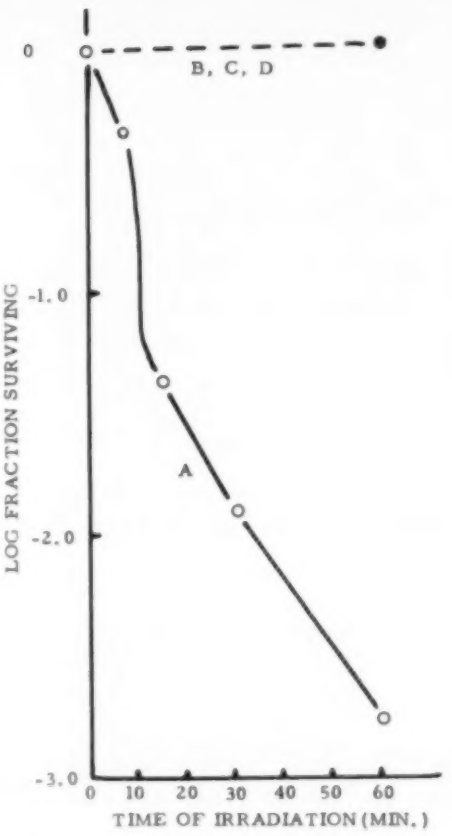

Fig. 1.—Photosensitized killing of yeast in the presence of erythrosin. The yeast concentration was approximately 3×10^7 cells per ml. in 0.067 *M*, pH 7.2 phosphate buffer and the erythrosin concentration was 1.14×10^{-6} *M*. *A*, Yeast plus dye in light; *B*, yeast minus dye in light; *C*, yeast plus dye in dark; *D*, yeast minus dye in dark.

The abilities of various substances to prevent the photosensitized killing of yeast are given in Table I, and typical experimental results showing the protective action of cysteamine are given in Table II. The results obtained with cysteine, 2-aminoethylisothiouronium bromide HBr, penicillamine, 1-mercapto-2-aminobutane, N,N-diethylmercaptoethylamine, and mercaptopropylamine were similar to those shown in Table II except that the degree of protection differed, as shown in Table I. Ascorbic acid in the highest concentration used, caused a drop in viable count of controls which varied from 51 to 72%, distinct from the light effects and apparently indicated some type of toxicity to the yeast under the test conditions. The other substances were nontoxic in the concentrations used.

The radioprotective properties of cyanide, cysteamine, cysteine, cystamine, 2-aminoethylisothio-

TABLE I.—ABILITY OF VARIOUS SUBSTANCES TO PROTECT YEAST AGAINST PHOTOSENSITIZED KILLING[a]

Substance	Final Concentration, *M*	Survival of Yeast, % Control Suspensions	Protected Suspensions
Glucose	0.028	0.5	0.75
	0.056	0.5	0.38
Potassium cyanide	0.016	0.72	0.98
	0.032	0.72	0.28
Ascorbic acid	0.011	0.54	1.73
	0.023	0.54	2.17
	0.046	0.5	21.8
Sodium thioglycollate	0.009	0.2	0.15
	0.017	0.2	0.125
β-Mercaptopropionic acid	0.009	0.42	0.55
	0.019	0.42	0.61
Mercaptosuccinic acid	0.01	1.3	0.33
	0.02	1.3	0.48
α-Monothioglycerol	0.02	0.33	0.58
Cysteamine HCl	0.004	0.58	88.6
	0.009	0.58	95.8
Cysteine HCl	0.004	0.75	5.7
	0.008	0.75	100.0
Cystamine HCl	0.009	0.74	0.52
	0.022	0.74	0.89
2-Aminoethylisothiouronium bromide HBr	0.004	0.13	30.3
	0.011	0.13	55.0
	0.018	0.13	55.0
Glutathione, reduced	0.004	0.36	1.45
	0.01	0.36	15.6
	0.02	0.36	80.0
Penicillamine HCl	0.02	0.31	32.2
1-Mercapto-2-aminobutane HCl	0.02	0.31	16.4
N,N-Diethylmercaptoethylamine HCl	0.02	0.31	39.3
Mercaptopropylamine HCl	0.02	0.31	2.5

[a] The reaction mixtures contained approximately 3×10^7 yeast cells per ml. at zero time, erythrosin, 1.14×10^{-5} *M*, the added substance where indicated, and enough 0.067 *M*, pH 7.2 phosphate buffer to make a total volume of 5 ml. Irradiation was carried on for sixty minutes.

uronium bromide HBr, glutathione, and N,N-diethylmercaptoethylamine have been reported in the literature (8, 23–28) and compounds of the structure of penicillamine, 1-mercapto-2-aminobutane, and mercaptopropylamine would be expected to be radioprotective (29, 30). Glucose and ascorbic acid are reported as having slight radioprotective abilities (8) while sodium thioglycollate, β-mercaptopropionic acid, and mercaptosuccinic acid have been found to be incapable of protecting animals from ionizing radiation (8). α-Monothioglycerol was included as an example of an —SH type compound lacking an amino group, which would not be expected to be radioprotective (29, 30). Several mercapto compounds were included to determine whether the —SH group alone protects against photosensitized killing and the results, although limited, would indicate that it does not. It is interesting to note that the highly protective compounds contained both an amino and —SH group. N,N-Diethylmercaptoethylamine, which was protective, has a substituted amino group. It is also of interest to note that compounds known to be highly protective against the biological effects of ionizing radiation were also protective against photosensitized killing, such as cysteine, cysteamine, 2-aminoethylisothiouronium bromide HBr, and glutathione. Cystamine, which protects only whole animals against ionizing radiation (8, 28), was inactive in protecting against photodynamic action.

TABLE II.—THE ABILITY OF CYSTEAMINE TO PROTECT YEAST AGAINST PHOTOSENSITIZED KILLING[a]

Conditions	Viable Yeast Cells per ml. after Irradiation
Yeats cells, in light	3.1×10^7
Yeast cells, in dark	3.2×10^7
Yeast cells, plus erythrosin, in dark	3.2×10^7
Yeast cells, plus erythrosin, in light	1.8×10^6
Yeast cells, plus erythrosin, plus cysteamine (final concn. 0.004 *M*) in light	2.75×10^7
Yeast cells, plus erythrosin, plus cysteamine (final concn. 0.009 *M*) in light	3.0×10^7

[a] The reaction mixtures, where indicated, contained 1.14×10^{-5} *M* concentration of erythrosin and the final volumes were made to 5 ml. with 0.067 *M*, pH 7.2 phosphate buffer. Irradiation was carried on for sixty minutes under the conditions described in the text.

The fact that the thiol type of radioprotective agents tested protected against photodynamic killing while the other radioprotective agents such as cyanide and glucose did not protect, coupled with the fact that β-mercaptopropionic acid, mercaptosuccinic acid, and thioglycollic acid, which are not radioprotective (8), did not protect against photodynamic killing would suggest the possibility of a relationship between the ability of a thiol compound to protect against ionizing radiation and photosensitized killing. The explanations for the effects of radioprotective agents have been of two sorts: (*a*) reduction in intracellular oxygen tension; and (*b*) combination with free radicals (31). The necessity for oxygen in photodynamic action has been well established (4) and recent work (32–34) has indicated that free radicals may be produced in the photochemical activation of dyes. Uri (35) and Blum and Kauzmann (16) have suggested the possibility that the photosensitized dye may be activated to a triplet state, which would react with oxygen to form a free radical. It may thus be possible that protection against photodynamic killing could be effected by neutralization by the protective substance of the above mentioned reactive species. It would appear that simple oxygen depletion is not the total answer.

An experiment was performed to determine the abilities of several of the compounds tested to reduce methylene blue. The latter was used in a concentration of 6.6×10^{-6} *M* while the compounds examined had a final concentration of 0.02 *M* except cysteine HCl which was 0.008 *M*, and the final volume of solution was made up to 5 ml. with pH 7.2, 0.067 *M* phosphate buffer. It was found that penicillamine reduced the methylene blue in less than five minutes and the dye remained reduced even after one hour on the shaker used in the irradiation experiments. Cysteine HCl and 1-mercapto-2-aminobutane HCl reduced the methylene blue in less than ten minutes and partial reoxidation

occurred when the mixtures were shaken. Cysteamine HCl slightly decolorized the methylene blue by thirty minutes and completely by two hours, while glutathione began to decolorize in forty-five minutes and completely decolorized by two hours. N,N-Diethylmercaptoethylamine HCl caused partial decolorization in two hours but 2-aminoethylisothiouronium bromide HBr and mercaptopropylamine HCl did not decolorize the dye by five hours. Thus, while all of the above compounds were capable of protecting yeast against photosensitized killing, they varied greatly in their ability to reduce methylene blue in unshaken solutions. Other possibilities include protection in some manner of susceptible sites on the yeast cell against the excited chemical species. Such a mechanism has also been suggested by Eldjarn and Pihl (36) who believe that cystamine and cysteamine form —S—S bonds with —SH groups of vital molecules, and thus protect these —SH groups from free radicals formed in an aqueous medium by ionizing radiation. Further studies to determine whether triplets and/or free radicals are formed under actual conditions of photosensitized killing would be helpful in deciding the validity of the above postulations.

The interesting correlation of the abilities of certain of the compounds investigated to protect against both photodynamic killing and damage due to ionizing radiation might suggest a possible use of the above procedures as a preliminary screen for compounds protecting against ionizing radiation and also suggests that the mechanism of the biological damage caused by both could be similar at some stage.

Finally, it would be of great interest to determine whether compounds protecting against lethal photosensitization of yeast would also protect experimental animals against photosensitization induced by such drugs as chlorpromazine and promethazine. Such studies are in progress.

SUMMARY

The photosensitized killing of *Saccharomyces cerevisiae* was used as an experimental model for studying photochemically induced biological damage and to permit screening of various compounds for their ability to prevent the photochemically induced effects. *S. cerevisiae*, in the presence of erythrosin as a photosensitizer, is rapidly killed when irradiated with white light. While it is known that reducing agents will prevent photodynamic killing, it appeared to be of interest to determine whether compounds known to protect against ionizing radiation would also prevent photodynamic effects. Of the compounds tested, cysteamine, glutathione, penicillamine, N,N-dimethylmercaptoethylamine, 1-mercapto-2-aminobutane, mercaptopropylamine, and 2-aminoethylisothiouronium bromide HBr (AET) protected yeast against photosensitized killing as did cysteine, but cystamine, potassum cyanide, glucose, β-mercaptopropionic acid, mercaptosuccinic acid, α-monothioglycerol, and sodium thioglycollate did not protect. Ascorbic acid protected at higher concentrations. Protection was dependent on the presence of the compound during irradiation. On the basis of the data presented, it appears that thiol type compounds which protect against the biological effects of ionizing radiation also protect yeast against photosensitized killing.

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Application of Neutron Activation Analysis to the Microanalysis of Gold-Containing Pharmaceuticals in Biological Materials

By JAMES P. VACIK and JOHN E. CHRISTIAN

A neutron activation analysis procedure was designed for the microanalysis of gold-containing compounds, which eliminates all prior chemical separation and permits direct qualitative and quantitative analysis for gold in a γ-ray scintillation spectrometer. The method was further applied to the determination of gold in biological tissues at the 5 to 60 mcg. level following administration of gold thioglucose.

UNTIL RECENTLY, the determination of gold in biological materials has not often been required; thus, few accurate methods of analysis have been developed and published. With the current widespread therapeutic use of radioactive gold (Au^{198}) and with the increasing therapeutic treatment with gold salts, a rapid and sensitive method is needed for the determination of the gold content of various biological materials. In addition, a sensitive method would be useful in studies of the uptake, metabolism, and determination of the overall distribution of gold-containing pharmaceutical products in biological systems.

It was the intent of this project to study and develop a rapid and sensitive technique of activation analysis applicable to biological samples which would eliminate the tedious and time consuming chemical separations previously employed (1–3) by others and permit direct counting and assay of irradiated samples.

Neutron activation analysis was chosen for this work due to its simplicity and extreme sensitivity. Sensitivities in the order of 0.003 mcg. may be obtained with gold from activation in a nuclear reactor at a flux of 5×10^{11} n/cm.^{2}/sec. (4). The method is simple, involving merely the placing of a sample to be analyzed and a suitable standard in a neutron flux for a sufficient time to produce a measurable amount of a radioisotope of the element being determined. The characterization of the radioactivity produced then serves as a qualitative tool; while the measurement of the intensity of the induced radioactivity in the sample is directly proportional (other factors being equal) to the amount of element present, thus furnishing a quantitative measurement independent of the state of chemical combination of the element in question (5).

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EXPERIMENTAL

Method of Counting.—The radioactivity resulting from all samples during this study was determined using a γ-ray scintillation spectrometer. This spectrometer consisted of a sodium iodine thallium-activated crystal well type detector,[1] which was coupled to a photomultiplier tube and an amplified circuit, a single-channel pulse height analyzer,[2] a scaling device,[3] an electronic sweep,[4] and a Leeds and Northrup 10 mv. strip chart recorder.

With this type of equipment it was possible to count gamma peaks with set window widths, to count manually the complete gamma spectra from 0 to 1 Mev., or to graph the complete gamma spectra automatically over set time intervals. With the shield employed on the scintillation detector it was possible to maintain an average unloaded background of 30 c.p.m. at the 0.411 Mev. gamma peak of Au^{198}.

The unit was calibrated with Cs^{137} and Co^{60} standard samples at reasonable intervals so that finding any specific photopeak of known energy was merely a matter of instrument adjustment. Counts under a particular photopeak could also be totaled on a register, thus allowing quantitative results with these totals, with peak heights, or with integrated areas under graphed photopeaks.

A gold^{198} spectrum was carefully plotted with the recording γ-ray spectrometer (see Fig. 1) in order to observe its characteristic shape and peak energy.[5]

Method of Neutron Irradiation.—The source of neutrons for sample activation of all biological materials was the Argonaut reactor (6) at the Argonne National Laboratories. Fifteen of the available experimental ports in this reactor are located in a graphite thermal column, 5 feet wide × 5 feet long × 4 feet high. It was in one of the 15 ports (specifically the J-10 stringer) where samples to be activated were placed. The J-10 stringer consists of an aluminum tray 4 inches × 4 inches × 5 feet long in which varying thicknesses of graphite blocks may be placed. This stringer at its closest end extends to within 4 inches of the reactor core. It was at this "closest end" where the 3 $^{15}/_{16}$ in. × 3 $^{15}/_{16}$ in. plexiglas holders (to be subsequently described) containing the samples to be irra-

[1] Serial K-40, Harshaw Crystals Inc., Cleveland 6, Ohio.
[2] Model 115, Radiation Instrument Development Laboratories, Chicago, Ill.
[3] Model 200-T, Radiation Instrument Development Laboratories, Chicago, Ill.
[4] Model 39-1, Radiation Instrument Development Laboratories, Chicago, Ill.
[5] Material obtained from Oak Ridge National Laboratories, Oak Ridge, Tenn.

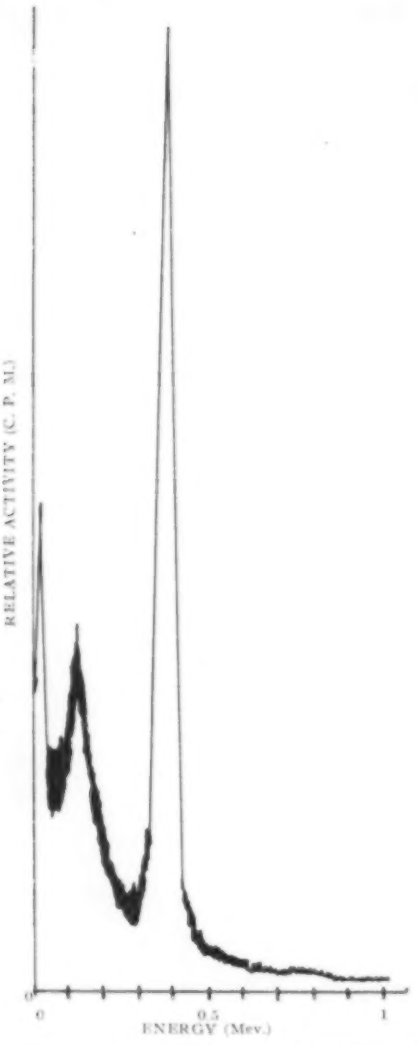

Fig. 1.—A γ-ray spectrum of a pure gold^{199} sample obtained from Oak Ridge National Laboratories.

diated were placed. The approximate flux at this point was $2 \times 10^9 n/cm.^2/sec.$ at the reactor setting employed.

The approximate neutron flux and the one-hour exposure time chosen for biological experimentation were arrived at by calculation of the radioactive growth and decay rates for gold^{198}, sodium[24], copper[64], and phosphorus[32]. The gold isotope is the one to be analyzed and the others are the most likely isotopes to be formed during tissue bombardment. Calculations showed that if the flux and time of bombardment were in the order of $10^9 n/cm.^2/sec.$ and one hour, respectively, the only normally occurring tissue components which could conceivably cause interference with the gold^{198} assay was the fifteen hour sodium[24]. Experimental comparison of qualitative spectra run twelve hours following bombardment with spectra of known gold revealed that all activity present in a biological sample irradiated at this flux was due only to gold^{198}. To avoid coincidence errors and correction factors, all samples counted were below 50,000 c.p.m. activity.

Irradiation Containers and Sample Tubes.—A sample holder was designed using a piece of plexiglas[6] ($3^{15}/_{16}$ in. $\times$ $3^{15}/_{16}$ in. $\times$ $^5/_8$ in.). This holder was made to fit into the aluminum tray of the J-10 stringer at the core end in the place of one of the carbon blocks. Thus the face of the holder was in a plane parallel to the core face. Six holes ($2^1/_2$ in. deep) were bored into the $^5/_8$ in. edge of the holder with a $^7/_{16}$ in. drill bit in order to accommodate six sample tubes. The tubes in which the samples were placed were composed of polyethylene tubing[6] ($^1/_4$ in. i. d. $\times$ $^3/_8$ in. o. d. $\times$ $1^1/_2$ in. long) closed at the bottom with solid plugs of $^1/_4$ in. polyethylene rod which was sealed in place by rotating the end of the tube against the hot tip of an electric soldering gun. The sample was then placed in the tube and the tube sealed with polyethylene in this same manner. Segments of polyethylene tubing $^1/_2$ in. long and $^3/_8$ in. o. d. were inserted over each sample tube in the holder to hold it in place and to fill the void above each. The holders were then closed with a narrow strip of adhesive tape.

In subsequent experimentation, two sample tube holders each containing six samples were placed side by side and irradiated at the same time in the J-10 stringer, thus allowing a total of 12 samples per bombardment. The initial run to determine any variation in neutron flux was made with twelve samples each containing 10 lambda of a standard gold chloride[7] solution and 240 lambda of liver tissue homogenate. The results indicated a deviation in c.p.m./mg. of only 2.1%.

To verify the relationship between radioactivity induced and the gold concentration in tissue, various quantities of gold chloride each contained in 10 lambda of water were placed into 240 lambda of liver tissue homogenate to make up a series of varying and known concentrations of gold in tissue. These samples were bombarded for one hour at a flux of $2 \times 10^9 n/cm.^2/sec.$ and then counted at the 0.411 Mev. peak of gold^{198} with a 3-volt window. The activity of the samples was plotted against the gold content in mcg. A plot of the resulting data yields a linear relationship, illustrating that the induced activity from gold in tissue is proportional to the concentration. Thus, an accurate method of assay for gold in biological materials without chemical processing was possible using the method described.

In order to illustrate the practicality of this assay procedure, a gold-containing pharmaceutical used in current therapy, gold thioglucose, was next employed in place of gold chloride. Samples of gold thioglucose containing 5, 10, 20, 30, 40, 50, and 60 mcg. of gold, respectively, and contained in 10-lambda volumes were made up to 500-lambda volume with liver tissue homogenate. As a standard to compensate for flux variations from holder to holder, gold chloride standards containing 325 mcg. of gold chloride in 10 lambda of distilled water plus 490 lambda

[6] Cadilac Plastic Corp., Chicago, Ill.
[7] Mallinckrodt Chemical Works, St. Louis 7, Mo.

of liver tissue homogenate were prepared and one placed in each sample holder. The samples and the standards were then irradiated for one hour at a flux of 2×10^9 *n*/cm.^{2}/sec. The activity was determined twelve hours after bombardment, corrected for decay, and, by means of the gold chloride standard, corrected to compensate for flux variations between holders and between different bombardments. The corrected activities were plotted against the known gold content of each sample to serve as a calibration curve for future work. A straight line relationship was seen to exist between the induced activity and the gold content, thus serving as a second check on the procedure.

TABLE I.—GOLD CONTENT OF VARIOUS ORGANS OF MICE THIRTY MINUTES AFTER THE INTRAPERITONEAL ADMINISTRATION OF GOLD THIOGLUCOSE[a]

Tissue	Radioactivity,[b] (c. p. m./Gm.)	Gold Present, mcg./Gm. of Tissue	Administered Dose/Gm. of Tissue, %
Adrenal glands[c]	17,794	250	0.08
Hypothalamic area[d]	1,196	20	0.01
Hypothalamic area[e]	1,513	20	0.02
Hypothalamic area[f]	1,410	20	0.03
Liver tissue[g]	5,470	80	0.14
Liver tissue[g]	3,334	50	0.08
Liver tissue[g]	4,808	70	0.10
Liver tissue[g]	4,564	70	0.11
Liver tissue[g]	3,414	50	0.08

[a] 1 mg./Gm. body weight.
[b] Samples were irradiated for one hour at 2×10^9 *n*/cm.^{2}/sec.
[c] Pooled adrenals of 15 animals.
[d] Pooled hypothalamic areas of seven animals.
[e] Pooled hypothalamic areas of five animals.
[f] Pooled hypothalamic areas of three animals.
[g] Pooled livers of three animals.

Using the relationship established between radioactivity and the known gold content of tissue, the gold content in various organs of mice, thirty minutes after the intraperitoneal administration of 1 mg./Gm. of gold thioglucose, was determined. Fifteen Rockland Swiss mice were used in the study. Thirty minutes after the administration of the gold thioglucose each animal was sacrified by decapitation and the liver, adrenals, and hypothalamic area of the brain removed. These organs were pooled, homogenized, and all sample volumes below 500 lambda made up to this volume with untreated homogenized tissue. The samples were then sealed in sample tubes and placed in holders along with one gold chloride (325 mcg./10 lambda) standard in 490 lambda of liver tissue homogenate per holder. The samples were irradiated in the J-10 stringer of the Argonaut pile for one hour at a flux of 2×10^9 *n*/cm.^{2}/sec. The samples were allowed twelve hours decay time following bombardment and were counted at the 0.411 Mev. peak of gold[198] with a 3-volt window to determine the amount of induced activity present. The concentration of gold present in each sample was determined by use of the calibration curve previously prepared. Qualitative spectra were also run at the end of twelve hours decay time and compared to the spectra of pure gold[198] to show that the only activity present was due to that of the gold.[198]

The prepared samples along with their activities and gold content are listed in Table I.

DISCUSSION

The activation analysis procedure described in this paper should be applicable to numerous problems arising in pharmaceutical and medicinal research. By increasing the neutron flux or time of bombardment by a factor of from two to five times, mounts of gold in quantities as low as 1 mcg. should be capable of being assayed without interference from beta-emitting isotopes and thus without chemical manipulations of any kind. The method should be adaptable to the assay of gold in the animal body, and to the identification of gold metabolites. In the latter instance, if known gold compounds and unknown tissue metabolites are chromatographed together and these chromatograms are irradiated, qualitative identification as well as quantitative evaluation may be accomplished. Histological slides of gold-containing tissues might be irradiated and audioradiographs prepared for study of tissue distribution. The procedure should prove to be a useful tool in the analysis of gold-containing pharmaceutical products.

SUMMARY AND CONCLUSIONS

1. A microgram-sensitive method of activation analysis of gold contained in biological samples was developed which does not require chemical separation or treatment of the samples either prior to or following neutron irradiation.

2. The method was established at 5 to 60 mcg. levels of gold in tissue; however, calculations indicate that by increasing the flux or the time of neutron exposure by a factor of from two to five times, amounts of gold as low as 1 mcg. should be capable of assay without chemical processing of tissue samples.

3. The method of assay described has been applied successfully to the determination of the gold content of the various organs of mice, thirty minutes after the intraperitoneal administration of gold thioglucose.

4. The method should be applicable with advantages to the numerous problems arising in pharmaceutical and medical practice and research requiring the assay of gold compounds contained in simple solutions or in biological materials.

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Interfacial Properties of Powdered Material; Caking in Liquid Dispersions I

Caking and Flocculation Studies

By BERNARD A. HAINES, Jr.†, and ALFRED N. MARTIN

Surface characteristics of particles and the intensity of particle interactions are presented as factors which may lead to caking of insoluble particles in aqueous dispersion. The degree of flocculation and the sediment volume were used as indexes of caking for sulfamerazine and bismuth subnitrate dispersions. Controlled particle flocculation by the addition of selected electrolytes produced a large volume of sedimented particles free from caking tendencies.

WHEN DISPERSED particles form into a compact mass at the bottom of the container, the suspension is said to have caked. Since the cake may contain the active ingredients, uniform dosage distribution cannot be assured even after vigorous shaking. Therefore, minimizing this condition is a necessary step in the compounding of suspensions. Although caking is a major problem to the manufacturing pharmacist in formulating mixtures, magmas, and lotions, little or no work relative to caking, as such, has appeared in pharmaceutical literature. For this reason the present study was undertaken.

The study of the principal factors leading to caking and flocculation has not progressed to a point where broad generalizations can be made. However, some underlying factors have been found to influence them.

The nature of the particle surface greatly influences the network structure which is produced in the cake or floccule. Rehbinder and Segalova (1) stated that two types of network structures may be formed in the sediments or cakes of suspensions and pastes: the condensation or strongly bonded type and the coagulation or weakly bonded type. A third type of particle interaction of the weak van der Waals type does not result in caking but rather leads to flocculation.

Physical and/or chemical reactions form strong bonds between the particles. Physical bonding may be brought about by crystal formation, which depends upon the degree of solubility of the dispersed material in the vehicle. At the points of contact between microcrystals, a thin layer of supersaturated solution exists. The smaller crystals slowly dissolve and cause the larger crystals to grow. This phenomenon, known as Ostwald ripening (2), leads to coarsening of the sediment and, in some instances, according to Kolthoff and Noponen (3), may also result in caking. The degree of caking is increased by a reduction of crystal size, which produces an increased number of crystalline points of contact. Furthermore, chemical bonding due to hydration effects may cause the porous particles to cement together into a hard crystalline mass. Husa (4) noted this effect in an aqueous dispersion of magnesium oxide and sodium bicarbonate. In preliminary tests, the writers found that if equal quantities of magnesium oxide and sodium bicarbonate were poured as a slurry into a mold and subjected to the A. S. T. M. (5) cement test, more than 200 pounds per square inch of pressure were necessary to break the resulting cake.

Coagulation or weakly bonded types of interaction also occur. Adhesion of particles by molecular interaction at points of contact forms weak bonds between the particles. The adhesion is due to a squeezing out of the dispersion medium at the points of contact, as in syneresis. The resulting residual layer of the dispersion medium prevents a strong molecular interaction at the points of contact. The strength of the cake which is formed depends upon the number of particles occupying a given volume. The particles may orient themselves in the sediment and, under the influence of gravity, coagulate in an arrangement of closest packing. Various clays, such as bentonite, exhibit this type of sediment.

Particles in dispersion with a high net charge density exhibit electrostatic forces of repulsion. When the forces of repulsion are greater than

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† Present address: The Squibb Institute for Medical Research, New Brunswick, N. J.
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those of attraction, the particles remain apart. Overbeek (6) observed that the addition of electrolytes decreased the density of the ionic atmosphere surrounding the particle and caused the forces of repulsion to be effective at a shorter distance from the particle. Thus, when the magnitude of the van der Waals attraction forces exceeds that of the electrostatic repulsion forces, the particles come together. This type of particle interaction produces flocculated systems consisting of large fluffy clumps of particles loosely held together, which do not form a hard sediment or cake.

Particle interaction leading to caking or flocculation may then be classified according to the intensity of the bonding at the points of contact. The condensation or strongly bonded type forms compact crystal aggregates or hydrates, which are irreversibly dispersed. The coagulation or weakly bonded type results from molecular interaction and can often be redispersed by vigorous mechanical agitation. The type of particle interaction leading to floccule formation is characterized by ease of redispersion and absence of caking. The loose irregular cluster formations break up when disturbed, but reform immediately after agitation ceases.

In the colloid literature, the word "stable" as applied to colloidal sols or suspensions is synonymous with "deflocculated." A flocculated suspension, consisting of large fluffy clumps which settle rapidly, is considered as "unstable." Accordingly, the industrial chemist and pharmacist have often concluded that a suspension should be maintained in the deflocculated condition. It is not generally recognized that deflocculated particles, which eventually do settle even in the presence of the most effective suspending agents, tend to form caked masses. Moreover, very frequently the suspending agent increases rather than prevents the caking in the sediment.

EXPERIMENTAL

Principles Employed.—In this investigation, a technique similar to that described by Fischer (7) is employed as an approach to the caking problem. It is possible to prevent or minimize caking by the addition of flocculating agents which tend to form loosely bonded networks in the suspension. These agents prevent the formation of condensation or coagulation structures and increase the redispersibility of the insoluble particles. The addition of regulated quantities of flocculating agents produces a controlled flocculation of the dispersed phase, the amount of agent depending upon the nature of both the particle and the agent added. The flocculating power of the agent is determined by the valence opposite in sign to that of the particle, and increases rapidly with an increase in the valence of the agent.

The volume to which the particles in suspension settle is known as the sedimentation volume. When compared with a nonflocculated suspension, a flocculated system will form a less dense sediment resulting in a larger sedimentation volume. The sedimentation volume is an important factor in determining the redispersibility of a suspension. Increasing the sedimentation volume by flocculation decreases the tendency of the dispersed particles to cake. The ultimate height, *Hu*, which provides a measure of the sedimentation volume to which a suspension will settle without agitation, is a function of the concentration of the suspension, the degree of flocculation, the particle size and, to some extent, the original height, *Ho*, of the suspension. Ward and Kammermeyer (8) studied the sedimentation rate and equilibrium sedimentation height of aqueous suspensions. They found that the relationship between weight concentration and the quantity *Hu/Ho* (ratio of ultimate height to original height of the sediment) could be represented by straight lines on log-log paper for definite particle size when the initial height was kept constant. From such a plot it is evident that sedimentation height is related to concentration by the equation

$$\log Hu/Ho = \log b + m \log c \quad \text{(Eq. 1)}$$

where b and m are arbitrary constants representing the intercept and slope of the logarithmic plot, respectively, and c is the weight concentration of the suspended particles per 100 ml. of suspension.

Procedure.—Aqueous dispersions of sulfamerazine and bismuth subnitrate were selected for this study, since they form a dense, difficultly redispersible cake. Powdered sulfamerazine U. S. P. was screened through a series of U. S. Standard sieves. The fraction used was that which passed through a No. 100 sieve and was retained on a No. 170 sieve. Bismuth subnitrate N. F. particles were classified by the sedimentation-elutriation method. Microscopic observation showed that both deflocculated powders consisted of particles within the size range of 2 to 10μ. Aqueous suspensions were prepared with various concentrations of powders, calculated to dry weight. Aerosol OT[1] was employed as a wetting agent for sulfamerazine to reduce the hydrophobic nature of the particles. The addition of the surface-active agent was found to have little or no effect on the recorded observations.

The suspensions were allowed to settle in 4- × 60-cm. calibrated, vertically mounted, glass sedimentation tubes held at ordinary room temperature. (Preliminary tests in a thermostat over a range of temperatures showed that room temperature was quite satisfactory for this work.) The sediment height was recorded at various time intervals until the ultimate sedimentation height, *Hu*, was reached.

RESULTS AND DISCUSSION

Sedimentation Studies.—When the particle concentration is such that a mutual interference in the motion of the particles reduces the velocity of sedimentation, the phenomenon known as hindered settling occurs. Aqueous sulfamerazine dispersions in concentrations of 1, 2, 5, 10, 15, and 20 Gm. per

[1] American Cyanamid and Chemical Corp., New York, N. Y.

100 ml. of dispersion were prepared and the rate of sedimentation was observed. As seen in Fig. 1, hindered settling was noted to occur at concentrations above 2 Gm. per 100 ml. of aqueous dispersion. When the data were replotted on a log-log grid in Fig. 2 in terms of *Hu/Ho* ratio *versus* particle concentration, a straight line relationship was obtained in good agreement with Eq. 1.

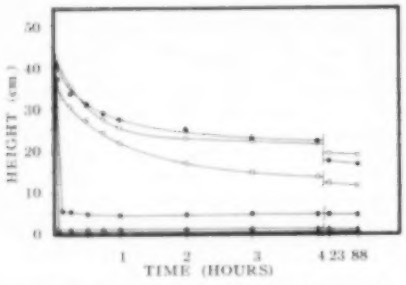

Fig. 1.—Sedimentation rate of sulfamerazine in various concentrations in distilled water (containing 0.1 Gm. Aerosol OT/100 ml. suspension). ▲, 1 Gm.; △, 2 Gm.; ■, 5 Gm.; □, 10 Gm.; ●, 15 Gm.; ○, 20 Gm. sulfamerazine/100 ml. suspension.

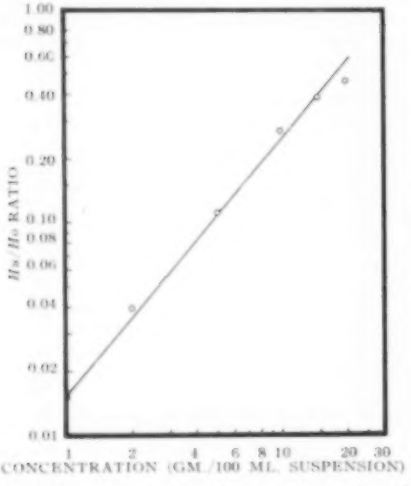

Fig. 2.—Sedimentation height ratio of sulfamerazine in various concentrations in distilled water (containing 0.1 Gm. Aerosol OT/100 ml. suspension).

Controlled Flocculation.—Two per cent suspensions of sulfamerazine and bismuth subnitrate were selected for the controlled flocculation studies, since at this low concentration hindered settling and viscosity effects do not influence the rate of sedimentation. The effects of various concentrations of anionic and cationic electrolytes on sedimentation height were observed. The addition of aluminum chloride hexahydrate to a sulfamerazine suspension caused the formation of large loose networks of floccules which were easily redispersed. The *Hu/Ho* ratio for sulfamerazine was large compared to the nonflocculated suspension, and there was no evidence of cake formation. The results are shown in Fig. 3.

The viscosity of the system was observed to rise sharply at high flocculent concentrations and the rise in *Hu/Ho* above 0.6 molar concentration of aluminum chloride was due to these effects. Disregarding viscosity effects, a continued decrease in *Hu/Ho* would be anticipated, which should eventually result in a caked condition as shown by the dotted line in Fig. 3.

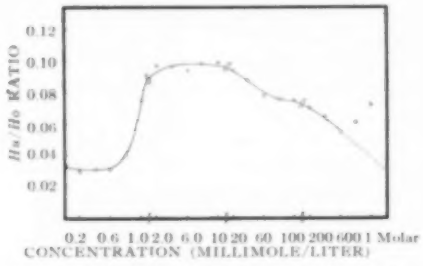

Fig. 3.—Sedimentation height ratio for 2 Gm./100 ml. sulfamerazine suspension flocculated with various concentrations of aluminum chloride.

The addition of monobasic potassium phosphate did not prevent caking in the sulfamerazine system. The *Hu/Ho* ratio of this sample was roughly the same as that for the nonflocculated suspension, and redispersion of the sediment was found to be difficult.

Bismuth subnitrate studies were made by methods similar to those of sulfamerazine with the omission of the wetting agent. Monobasic potassium phosphate was found to be a good flocculating agent, while aluminum chloride had no effect on the bismuth subnitrate. The results may be seen in Fig. 4.

Effect of Hydrophilic Colloids.—The effect of the addition of hydrophilic colloids was observed on sulfamerazine dispersions in the presence of aluminum chloride or monobasic potassium phosphate, as recorded in Tables I and II. It was found that at low concentrations, used to avoid viscosity effects, either Kelcoloid LV[2] or CMC[3] (sodium carboxymethylcellulose) did not enhance the sedimentation characteristics of the sulfamerazine dispersion. In fact, in the presence of the colloidal dispersants the addition of phosphate ions in some instances had an adverse effect on the *Hu/Ho* ratio when compared with the original nonflocculated aqueous suspension.

The floccule produced by aluminum chloride was quite stable, indicating that this preparation should have an extended shelf life. Prolonged, intermittent shaking in a mechanical shaking device had no effect on the sedimentation height, which indicated that the floccule was either not significantly broken up or was reformed as soon as the agitation was stopped. The suspension, flocculated with aluminum chloride, to which a small amount of

[2] Kelco Co., New York, N. Y.
[3] Type 70, medium viscosity; Hercules Powder Co., Wilmington, Del.

TABLE I.—FLOCCULATION OF SULFAMERAZINE U. S. P. IN THE PRESENCE OF KELCOLOID LV, CMC, AND/OR ALUMINUM IONS

Combination	Concn., Gm./100 ml. Suspension	$AlCl_3$ Molar Concn.	Hu/Ho Ratio	Comments
Sulfamerazine[a] Distilled water	2.00	...	0.03	No flocculation, difficult to redisperse, caked.
Sulfamerazine[a] Kelcoloid LV	2.00 0.02	...	0.03	No flocculation, redispersible, soft cake formed.
Sulfamerazine[a] CMC	2.00 0.02	...	0.03	No flocculation, difficult to redisperse, caked.
Sulfamerazine[b] $AlCl_3 \cdot 6H_2O$	2.00	0.001 0.003 0.004	0.08 0.10 0.10	Large floccule formation, easily redispersed, no caking.
Sulfamerazine[b] LV Kelcoloid $AlCl_3 \cdot 6H_2O$	2.00 0.02	0.001 0.003 0.004	0.02 0.06 0.05	Floccule formation, easily redispersed, soft cake formed.
Sulfamerazine[b] CMC $AlCl_3 \cdot 6H_2O$	2.00 0.02	0.001 0.003 0.004	0.09 0.10 0.09	Large floccule formation, easily redispersed, no caking.

[a] The suspension contained 0.1 Gm. Aerosol OT per 100 ml. [b] The suspension contained 0.2 Gm. Aerosol OT per 100 ml.

TABLE II.—FLOCCULATION OF SULFAMERAZINE U. S. P.[a] IN THE PRESENCE OF KELCOLOID LV, CMC, AND/OR PHOSPHATE IONS

Combination	Concn., Gm./100 ml. Suspension	KH_2PO_4 Molar Concn.	*Hu/Ho* Ratio	Comments
Sulfamerazine Distilled water	2.00	...	0.03	No flocculation, difficult to redisperse, caked.
Sulfamerazine KH_2PO_4	2.00	0.001 0.004 0.007	0.03 0.03 0.02	No flocculation, difficult to redisperse, caked.
Sulfamerazine Kelcoloid LV KH_2PO_4	2.00 0.02	0.001 0.004 0.007	0.03 0.03 0.02	No flocculation, difficult to redisperse, caked.
Sulfamerazine CMC KH_2PO_4	2.00 0.02	0.001 0.004 0.007	0.02 0.03 0.03	No flocculation, difficult to redisperse, very hard cake formed.

[a] The suspension contained 0.1 Gm. Aerosol OT per 100 ml.

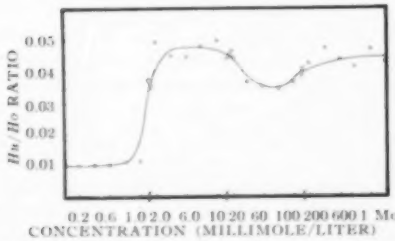

Fig. 4.—Sedimentation height ratio for 2 Gm./100 ml. bismuth subnitrate suspension flocculated with various concentrations of monobasic potassium phosphate.

CMC was added as a suspending agent, also exhibited a stable floccule structure. The well-flocculated suspensions did not adhere to the sides and bottom of the containers on simple inversion, and no evidence of caking could be observed over a period of three years. However, the concentration of CMC or Kelcoloid was too low to suspend the product adequately. When the concentration of the hydrophilic colloid was adequate to suspend the sulfamerazine dispersion, the aluminum ions formed stringy masses of colloidal material that made the product pharmaceutically unacceptable.

SUMMARY

The study demonstrates that a suspension in a state of fine deflocculated particles leads to, rather than obviates, caking. Moreover, moderate amounts of some commonly used suspending agents and electrolytes exaggerate, rather than prevent, caking. The addition of Kelcoloid, CMC, and phosphate ions had such an effect on aqueous sulfamerazine dispersions in the absence of flocculating agents. This study suggests that controlled flocculation, rather than defloc-

culation, will prevent caking in some pharmaceutical suspensions. Small amounts of nontoxic flocculating agents, such as aluminum chloride and monobasic potassium phosphate, may be employed to partially flocculate sulfamerazine and bismuth subnitrate suspensions and form loose clusters of particles that do not cake.

However, the addition of suspending agents such as CMC, most of which are negatively charged, cannot be added to stabilize the sulfamerazine suspension flocculated with aluminum chloride. The aluminum ions react with negatively charged viscous suspending agents and form a precipitate of strings and clumpy masses. On the other hand, the addition of a suspending agent to the bismuth subnitrate particles flocculated with phosphate ions shows no such incompatibility. This has already been observed by Schmitz and Hill (9) and Husa and Plaxco (10). Further work must be done to overcome these difficulties.

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Evaluation of a Sustained Release Form of Phenylpropanolamine Hydrochloride by Urinary Excretion Studies

By K. R. HEIMLICH, D. R. MacDONNELL, T. L. FLANAGAN, and P. D. O'BRIEN

The sustained releasing properties of phenylpropanolamine hydrochloride contained in Spansule capsules were evaluated by conducting urinary excretion studies. The average maximum rate of excretion after a unit dose of unprocessed drug was used as a basis of comparison for two sustained release dosage forms. The data on the sustained release preparations were also evaluated statistically.

RECENTLY, several studies have been reported in the literature in which urinary excretion data have been employed for the evaluation of sustained release dosage forms (1–5). The design of the experiments and the use of the data collected in these studies have varied somewhat with each investigation, depending on the type of drug used and the purpose of the study. In most cases, the extent to which urinary excretion data may be used in the evaluation of sustained dosage forms will depend upon the specificity of the quantitative method, the metabolic fate of the drug in the body, and the relationship between pharmacological response and the level of the drug in the blood or tissues.

Sustained release forms of drugs which are excreted completely and essentially unchanged and for which the drug level in the blood may be directly related to response afford a straightforward and precise evaluation of the prolonged action (1). However, this direct evaluation is not always possible, since some compounds undergo complicated detoxification or recycling processes, and it may not be practical to use an analytical procedure which will distinguish between unchanged drug and metabolites. Also, the level of the drug in the blood may not bear a direct relationship to the pharmacological re-

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sponse. In these instances, it is necessary to evaluate the sustained release characteristics of the dosage form by a relative comparison with the unprocessed drug. These comparisons may be accomplished by determining the amount of drug excreted in the urine after the administration of a sustained release dose and after multiple doses of unprocessed drug (6), or by comparing the urinary excretion rate over the claimed period of action after the administration of a sustained release dose with the average maximum excretion rate after administration of a unit dose of unprocessed drug (3).

The present study deals with the evaluation of a sustained release form of phenylpropanolamine hydrochloride in the following Spansule[1] capsule formulation:

phenylpropanolamine hydrochloride	50 mg.
chlorpheniramine maleate	8 mg.
isopropamide (added as the iodide)	2.5 mg.

The evaluation was carried out on the phenylpropanolamine hydrochloride component since the sustained release properties of chlorpheniramine maleate in a similar dosage form were previously determined (7, 8). Isopropamide, being an inherently long acting drug, was not put into sustained release form. The Spansule capsule dosage used in the tests was three times that recommended for the commercial product to facilitate the analytical procedure.

The evaluation was conducted in a series of three tests. The first was carried out to determine the excretion characteristics of the unprocessed drug, the second to obtain excretion data after the administration of two different sustained release formulas, and the third to test the excretion after the administration of divided doses of unprocessed drug.

EXPERIMENTAL

Preparation of Dosage Forms.—The general procedure used in the preparation of the sustained release material was that given by MacDonnell (9). The chlorpheniramine maleate and phenylpropanolamine hydrochloride were contained on a single population of pellets to which were applied varying amounts of a slowly digestible sustained release coating. The various sustained release groups thus prepared were blended in amounts calculated to obtain materials with two different *in vitro* release rates. The blends were then mixed with nonsustained release isopropamide pellets and encapsulated. The *in vitro* release rates of the chlorpheniramine maleate and the phenylpropanolamine hydrochloride were determined using the apparatus and general method reported by Souder and Ellenbogen (10). The percentage of drug released at specified times was obtained by difference after spectrophotometrically determining the amount of drug in the undisintegrated pellets remaining in the apparatus. The *in vitro* release rates of the two formulations, formula F and G, after encapsulation are given in Table I.

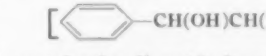
TABLE I.—*In vitro* RELEASE RATE OF PHENYLPROPANOLAMINE HYDROCHLORIDE AND CHLORPHENIRAMINE MALEATE FROM SPANSULE CAPSULES

		Per Cent Drug Released per Hourly Intervals			
Formula	Drug	0[a]	1/2 + 1	1 1/2 + 3	3 1/2 + 5 1/2
F	P[b]	34	56	79	88
	C[c]	41	62	82	88
G	P	4	12	29	43
	C	5	20	30	50

[a] Time in simulated gastric fluid T.S. U. S. P. XV, and simulated intestinal fluid T.S. U. S. P. XV.
[b] Phenylpropanolamine hydrochloride.
[c] Chlorpheniramine maleate.

The nonsustained release forms of capsules used in the study were made by preparing lactose dilutions of the three drugs.

Analysis of Specimens.—Sympathomimetic amines have been determined in biological media by various investigators (11–14); however, the methods were either nonspecific or time consuming. The oxidation of amino acids containing a hydroxyl and an amino group on adjacent carbon atoms has been reported (15–17). Since phenylpropanolamine



also contains the adjacent hydroxyl and amino groups in the side chain, the oxidation to benzaldehyde with subsequent extraction and spectrophotometric analysis appeared to be a convenient method of analysis.

The analyses were carried out by pipetting 0.5 ml. of each urine specimen into each of two 40-ml. centrifuge tubes. One of these tubes was designated as the blank. Water in the amounts of 1.5 ml. and 0.5 ml. was added to the blank and test samples, respectively, followed by one drop of phenolphthalein indicator. The solutions were made basic to the indicator by adding 1 *N* sodium hydroxide in a dropwise manner. Then 1 ml. of 0.075 *M* sodium periodate was added to the test sample. The samples were agitated and set aside for ten minutes. After the addition of 10 ml. of ether to each tube, they were placed on an automatic shaker for ten minutes. The ether phases were transferred to clean 40-ml. centrifuge tubes containing 5 ml. of 0.1 *N* sodium hydroxide and shaken for five minutes. The ether layers from the oxidized and blank samples were transferred to 1-cm. glass stoppered cuvettes and read in an ultraviolet recording spectrophotometer. The test sample was read against the blank over the range of 220 to 280 mμ, and the absorbance at 266 mμ (trough) subtracted from the absorbance at 242.5 mμ (peak) to obtain the absorbance from benzaldehyde in the oxidized sample. This value was converted to concentration of phenylpropanolamine hydrochloride by means of the standard curve shown in Fig. 1.

Clinical Studies.—In the first test three healthy, adult subjects were given 50 mg. of phenylpro-

[1] Smith Kline and French brand of sustained release compositions.

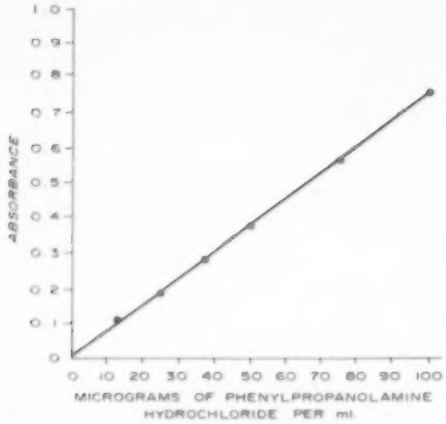


Fig. 1.—Standard curve for phenylpropanolamine hydrochloride in water.

panolamine hydrochloride, 8 mg. of chlorpheniramine maleate, and 2.5 mg. of isopropamide as the unprocessed drug (formula H) in a unit dose. All dosage forms were administered one hour before meals. Urine samples were collected as indicated in the tabulated data given in Table II.

In the second test, five subjects were given 150 mg. of phenylpropanolamine hydrochloride and 24 mg. of chlorpheniramine maleate in sustained release form and 7.5 mg. of isopropamide in nonsustained release form as 3 Spansule capsules (formula F). Also, six subjects were given the same dosage in the form of three slower *in vitro* releasing Spansule capsules (formula G). Specimen collections were made as indicated in Table III.

In the third test, nine subjects were administered 50 mg. of phenylpropanolamine hydrochloride, 8 mg. of chlorpheniramine maleate, and 2.5 mg. of isopropamide, all in the unprocessed form (formula H). At four and eight hours after the initial dose, the subjects again ingested the same dosage. Thus, the total dose taken in three divided amounts was equal to that taken in sustained form in the second tests. Again urine specimens were collected for the time periods given in Table III.

RESULTS AND DISCUSSION

In the preparation of sustained release dosage forms the primary function of the *in vitro* release rate testing is to insure that each batch of material prepared is consistent with material which exhibited desirable *in vivo* characteristics. However, as more objective *in vivo* data are collected on various drugs in sustained release form, the method of *in vitro* testing used in this study (10) acquires more significance as a guide to formulation. It has been found that drugs which are freely soluble or soluble according to the U. S. P. classification and have pKa values of 8 to 10 have produced satisfactory sustained *in vivo* responses if their release *in vitro* has approximated the following release rate pattern:

Time, hr.	% Drug Released
1/2	20–40
2	40–60
4 1/2	60–80
7	>80

Also, materials which have had significantly different *in vitro* release rates have been shown to be unsatisfactory by objective *in vivo* tests (6). Based upon these facts, the sustained release form of phenylpropanolamine hydrochloride was prepared with the above release rates as the best estimate for a satisfactory product. The significantly slower material was prepared to determine if a decrease in the *in vitro* release rate would produce a proportionate decrease in the *in vivo* availability.

The metabolic fate of sympathomimetic amines varies with different compounds and with the test animal used. Richter (11) reported a 92% recovery of ephedrine in the urine after twenty-four hours. However, no distinction was made between ephedrine and norephedrine. No oxidation by amine oxidase was found. Axelrod (14) found small amounts of ephedrine to be excreted as *p*-hydroxyephedrine and *p*-hydroxynorephedrine. Also, *p*-hydroxyamphetamine has been shown to be excreted in the urine of dogs as a major metabolite of amphetamine (18).

It was assumed in the present study that if hydroxylation of the phenylpropanolamine ring did occur, the oxidation product of this hydroxylated metabolite would not be extracted into the ether phase in the analytical procedure. Thus, the benzaldehyde would result only from the oxidation of phenylpropanolamine. To test the validity of the assumption, two compounds, 2-amino-1-hydroxy-1-(4-hydroxyphenyl) propane hydrochloride and 2-amino-1-hydroxy-1-(3,4-dihydroxyphenyl) butane hydrochloride, were analyzed by the present method. The spectrophotometric curves obtained in these analyses are shown in Fig. 2. The plots indicated that a ring hydroxylated compound would not interfere with the analytical method. It was concluded from this that the compound being measured was the oxidation product of phenylpropanolamine, i. e., benzaldehyde.

Table II.—Excretion of Phenylpropanolamine Hydrochloride After the Administration of a Single 50-mg. Dose

		Drug Excreted per Time Period, mg.							
	Pre-treatment	Post-treatment						0–24	
Subject	−8–0 hr.	0–2 hr.	2–4 hr.	4–6 hr.	6–8	8–12 hr.	12–24 hr.	Total	%
E. L.	0	11.4	13.4	7.7	6.5	6.8	3.3	49.1	98.2
W. L.	1.1	16.6	10.9	6.5	4.1	4.0	3.1	45.2	90.4
K. L.	2.9	7.4	12.8	8.1	9.6	7.0	5.6	50.5	101.0
Mean	1.3	11.8	12.4	7.4	6.7	5.9	4.0	48.3	96.5

TABLE III.—EXCRETION OF PHENYLPROPANOLAMINE HYDROCHLORIDE AFTER THE ADMINISTRATION OF SUSTAINED RELEASE DOSAGE FORMS, MG.

Subject	Pre-treatment −8–0 hr.	Post-treatment 0–4 hr.	4–8 hr.	8–12 hr.	12–24 hr.	24–36 hr.	0–36 hr. Total, mg.	0–36 hr. Per Cent Dose
Formula F								
J. C.	0.2	30.1	24.2	26.7	29.2	31.8	142.0	94.7
C. N.	2.1	21.0	25.5	36.7	39.9	10.3	133.4	88.9
A. K.	0.1	26.0	19.2	22.6	28.7	27.0	123.5	82.3
W. S.	1.2	28.7	43.2	28.0	31.1	7.4	138.4	92.3
V. C.	1.5	33.5	46.0	30.7	27.4	6.3	143.9	95.9
Mean	1.0	27.9	31.6	28.9	31.3	16.6	136.2	90.8
Formula G								
J. O'N.	1.3	3.2	11.5	17.9	51.7	18.3	102.6	68.4
S. L.	1.4	9.6	16.8	22.7	40.7	12.0	101.8	67.9
C. L.	1.8	1.8	9.4	15.5	49.4	28.7	104.8	69.9
J. E.	0.2	2.3	13.4	13.4	25.6	17.6	72.3	48.2
W. B.	2.8	4.5	17.0	20.4	42.4	19.5	103.8	69.2
Mrs. W. B.	0.8	8.3	18.5	22.5	41.8	4.7	95.8	63.8
Mean	1.4	5.0	14.4	18.7	41.9	16.8	96.9	64.6

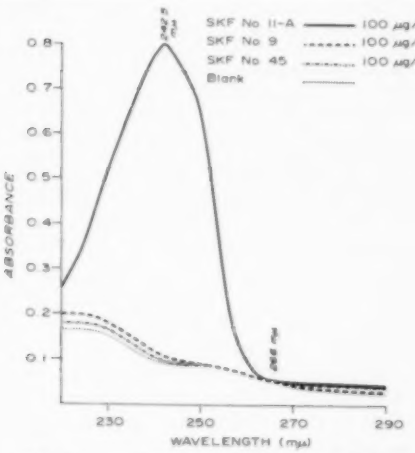


Fig. 2.—Spectrophotometric curves of ether extracts of SKF 11-A [phenylpropanolamine hydrochloride], SKF 9-A [2-amino-1-hydroxy-1 (3,4-dihydroxyphenyl) butane hydrochloride], SKF 45-A [2-amino-1-hydroxy-1-(4-hydroxyphenyl) propane hydrochloride], and a blank.

The amounts of phenylpropanolamine hydrochloride excreted in the urine after administration of a unit dose in the first test are given in Table II.

From the above data it is possible to calculate the biological half-life and the specific rate constant for excretion. Since the recovery of the drug was essentially complete, the data were plotted as per cent remaining in the body *vs.* time on semilogarithmic paper assuming first-order recovery. This plot is shown in Fig. 3. The specific excretion rate constant and the half-life were calculated from the plot using

$$k = -2.303 \cdot \text{slope} \qquad \text{(Eq. 1)}$$

and

$$t_{1/2} = \cdot 693/k \qquad \text{(Eq. 2)}$$

derived from first-order rate equations. These values are 0.18/hour and 3.9 hours, respectively.

These figures indicate that the drug is a good candidate for sustained release since a therapeutic blood level would be reduced by half in just 3.9 hours. Also, the data made possible a calculation of the amount of drug which should be used as a maintenance dose by employing the following

$$A = k \cdot b \cdot h \qquad \text{(Eq. 3)}$$

Where A is the maintenance dose, k is the specific excretion rate constant, b is the initial dose, and h is the number of hours for which action is desired. The excretion rate constant, k, is 0.18/hour (found from the slope of the plot in Fig. 3), b is 50 mg., and h is 10–12 hours. Solving the equation for A gives a required maintenance dose of 99 mg. Since for test purposes three times the initial dose was given, one-third this amount (approximately 33 mg.) was supplied in the commercial product.

The average maximum rate of excretion in the early periods from the subjects in Table II is approximately 7.1 mg./hour. The function of a sustained release dosage form is to duplicate as closely as possible this excretion rate for the time claimed. To determine this, the second test was carried out evaluating two sustained release forms having significantly different *in vitro* release rates (see Table I). The results are given in Table III.

In order to determine if a constant rate of excretion was obtained, the data in Table III were plotted accumulatively against time in Fig. 4. The relatively good linearity from the four to twelve hour period for the formula F Spansule capsule indicates a fairly constant rate of excretion. The value of 7.6 mg./hour calculated from the slope of the line compares favorably with the average maximum rate of 7.1 mg./hour obtained after administration of the unit dose and demonstrates that sustained release was established for the twelve-hour period. The formula G Spansule capsule was not considered satisfactory as demonstrated by lack of linearity in the accumulative plot and reduced physiological availability.

Another method of evaluation of sustained release dosage forms is a statistical comparison of the urinary excretion of the drug after administration of the sustained release form and after divided doses of the unprocessed drug. To demonstrate this, the unprocessed drug was administered every four hours for three doses in the third test. The excretion data are given in Table IV.

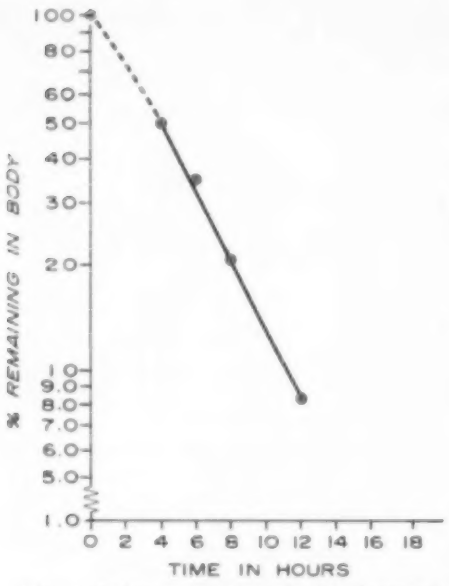


Fig. 3.—Urinary excretion of phenylpropanolamine hydrochloride after the administration of a unit dose of 50 mg. with 8 mg. of chlorpheniramine maleate and 2.5 mg. of isopropamide.

These data were compared statistically by analysis of variance with the data in Table III. The comparison is shown in Table V.

The significant value for the F test at the 95% confidence level for formulas is 4.45 and for formulas × times (release rate patterns) is 7.71. Thus, there are no significant differences in the excretion levels, patterns (formula × times) or the per cent of dose recovered after the administration of the formula F Spansule capsule and the formula H capsules (unprocessed drug) given every four hours for three doses. This is also reflected in the accumulative plots shown in Fig. 5. This plot also indicates that, although fairly good linearity was produced from the four to twelve-hour period for both forms, a true equilibrium state was not established. The slope of the unprocessed drug plot was found to be approximately 7.8 mg./hour. This value should have nearly equaled the input rate, since the recovery of the drug was found to be essentially complete.

The excretion levels, pattern, and per cent of dose recovered from the formula G Spansule capsule were significantly different from the other two formulations and were considered unsatisfactory.

These comparisons indicate that the formula F Spansule capsule can in all respects adequately replace multiple daily dosage of unprocessed drug. The data also indicate that sustained release formulations having distinctly different *in vitro* release rates as determined in this study can be readily differentiated in urinary excretion tests.

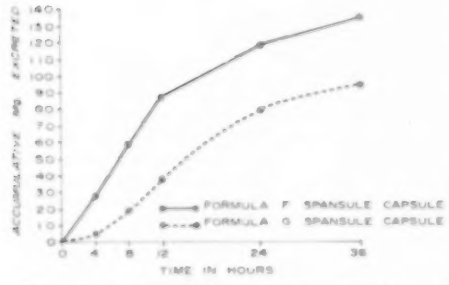


Fig. 4.—Accumulative urinary excretion of phenylpropanolamine hydrochloride after administration of sustained release capsules.

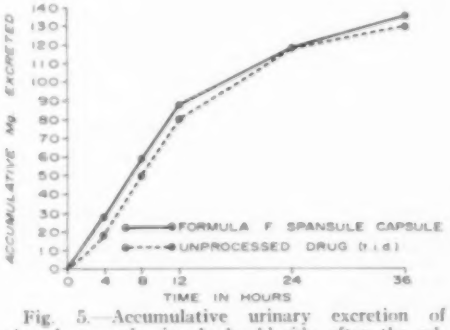


Fig. 5.—Accumulative urinary excretion of phenylpropanolamine hydrochloride after the administration of unprocessed drug (t.i.d.) and sustained release capsules.

Table IV.—Milligrams of Phenylpropanolamine Hydrochloride Excreted After the Administration of 50 mg. of Unprocessed Drug Every Four Hours for Three Doses

Subject	Pre-treatment −8–0 hr.	Post-treatment 0–4 hr.	4–8 hr.	8–12 hr.	12–24 hr.	24–36 hr.	0–36 hr. Total, mg.	0–36 hr. Per Cent Dose
J. E.	0.4	23.7	34.3	21.3	33.8	4.4	117.5	78.3
C. L.	0.4	15.4	30.1	28.7	39.9	8.4	122.5	81.7
S. M.	0.1	10.9	36.5	37.8	36.0	8.9	130.1	86.7
K. S.	0	8.5	25.3	37.8	41.0	8.3	120.9	80.6
G. C.	2.9	20.7	37.5	41.7	40.6	5.5	145.9	97.3
S. L.	0.8	21.7	37.3	40.4	35.6	5.8	140.8	93.9
B. J.	0.3	22.6	36.5	7.0	44.3	15.0	125.3	83.5
A. K.	0	19.6	22.4	30.0	32.2	26.9	131.1	87.3
C. N.	1.4	20.0	25.6	35.7	44.9	14.8	141.0	94.1
Mean	0.7	18.1	31.7	31.2	38.7	10.9	130.6	87.1

TABLE V.—ANALYSIS OF VARIANCE OF DATA FROM TABLES III AND IV

Source of Variation	*df*	*SS*	*MS*	*F*
Amounts Excreted				
Formulas	2	1094.52	547.26	
F *vs.* H	1	20.52	20.52	0.9
Other	1	248.22	248.22	11.4
Patients within	17	369.59	21.74	
Times	4	7153.35	1788.33	
Times × Formula	8	2699.139	337.392	
Time × (F *vs.* H)	4	581.38	145.34	2.41
Misc.	4	713.44	178.36	2.96
Residual error	68	4088.37	60.12	
Total	99	15404.97		
F *vs.* G	1	248.22	248.22	11.4
Time *vs.* F *vs.* G	4	1985.70	496.42	8.25
Per Cent of Dose				
Formulas	2	2430.11	1215.06	
F *vs.* H	1	45.82	45.82	0.9
Misc.	1	550.37	550.37	11.3
Patients within	17	824.76	48.52	
Total	19	3254.87		
F *vs.* G		1879.74	1879.74	38.7

SUMMARY

1. The urinary excretion rate parameters, specific excretion rate constant, and biological half-life for phenylpropanolamine hydrochloride were determined. The excretion data showed the drug to be rapidly and almost completely excreted and a good candidate for a sustained release preparation.

2. A sustained release preparation (formula F) judged by *in vitro* tests as adequate, maintained an excretion rate for twelve hours which compared closely to the average maximum excretion rate after ingestion of a single dose of unprocessed drug. A sustained release preparation purposely prepared so that it had a significantly slower *in vitro* release rate (formula G) proved to be unsatisfactory as shown by the urinary excretion pattern and the total availability.

3. A statistical comparison of urinary excretion after the administration of sustained release material and the unprocessed drug indicated that the Spansule capsule was in all respects equivalent to administering the unprocessed drug every four hours for three doses.

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Determination of the Hydrophile-Lipophile Balance of Acacia Using Various Surfactants

By WALLACE L. GUESS

The hydrophile-lipophile balance (HLB) of acacia has been determined in the presence of various ethylene oxide derivatives of sorbitan fatty acid esters. It was also found that different lots of acacia produced the same HLB values. A revised HLB value of 11.87 for acacia has been proposed.

MANY OF THE SYNTHETIC surfactants have been classified by the HLB system (1, 2), making the use of these products in pharmaceutical formulations simpler. The extension of the HLB system of classifying emulsifying agents to natural products has been recently reported (3, 4). However, it was felt that the HLB values reported in these papers were extremely low for such an efficient emulsifier as acacia. It was felt that many factors, including surfactant employed, conditions of agitation, and lot of acacia used could have been responsible for the value reported. Therefore, the work reported here was undertaken to determine (*a*) if a variation of surfactant would cause a change in the HLB of acacia, (*b*) if conditions of agitation exerted any influence on the HLB, and (*c*) whether there would be lot variations between various samples of acacia.

EXPERIMENTAL

In order to determine the HLB value of acacia, oil-in-water emulsions were prepared from a single lot of liquid petrolatum U. S. P. using acacia in conjunction with a synthetic surfactant of known HLB value.

Determination of HLB Value.—The method originated by Griffin (1) and modified by Chun, *et al.* (3, 4), was used in this study. A moisture determination on acacia by the toluene distillation method and by oven-heating at 100° was carried out in order to be assured of an accurate amount of acacia in each experiment. The proper quantity of acacia was weighed on an analytical balance and distributed in the oil phase. The calculated quantity of Tween[1] was added to the oil as an aqueous dilution containing 100 mg. Tween per ml. Distilled water was then added to make a total weight of 100 Gm. The final emulsion contained 50% liquid petrolatum.

Emulsification was effected by shaking on a Red Devil paint conditioner at 1,600 oscillations per minute. Each emulsion was shaken for five minutes immediately after mixing the components. A second five minutes of shaking was undertaken one hour later, and a final five-minute period of shaking was conducted at the end of twenty-four hours. The emulsions were allowed to stand undisturbed for twenty-four hours after the final shaking and evaluated. Subsequent experiments to evaluate conditions of agitation were carried out in a Waring Blendor, and by shaking by hand for a given period of time.

Evaluation of the Emulsions.—The emulsions were evaluated primarily on the basis of degree of creaming and oil separation. In almost every case, the best emulsion of a given series showed a lesser degree of creaming and oil separation than a poorer one in the series. Occasionally, where two good emulsions were almost identical, evaluation was on the basis of color and apparent globule size. All emulsions were evaluated by a "blind" technique and confirmed by another research worker.

Calculations.—When the optimum ratio of acacia to synthetic surfactant had been determined by the evaluation procedure, the HLB of acacia was calculated from the formula (3)

$$\text{HLB} = \frac{R - (H \times S)}{N}$$

where HLB is the calculated value of acacia, R is the "required HLB" of the liquid petrolatum, H is the HLB value of the Tween used, S is the per cent of Tween expressed as a decimal fraction, and N is the per cent of acacia expressed as a decimal fraction.

The revised HLB value of 12.0 for liquid petrolatum was used in all calculations.

RESULTS AND DISCUSSION

Over 100 emulsions were prepared and evaluated in the determination of the optimum ratio of acacia to synthetic emulsifier. Figure 1 is a typical example illustrating how the ratio of acacia to Tween 80 was successively decreased until the optimum was reached for liquid petrolatum. The emulsions corresponding to a given ratio of acacia to Tween 80 are illustrated with open circles. The final series of emulsions (series 5) covered the entire range of HLB values and confirmed the best ratio of emulsifiers.

The results of this study, covering three different lots of acacia and four different polyoxyethylene sorbitan fatty acid esters are shown in Table I.

It appears from the results shown in Table I that there is no significant difference in the HLB of acacia caused by the particular Tween being used. The reporting of an HLB value in the second decimal place is a questionable value, but is only shown in Table I to indicate the magnitude of the first decimal figure. It would also appear from an inspection of

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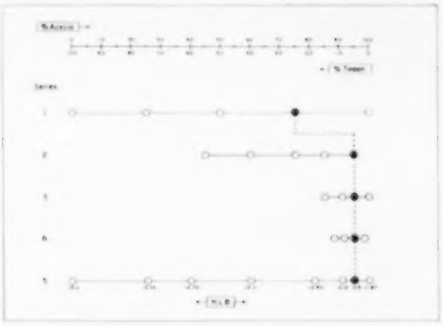

Fig. 1.—Flow diagram indicating method of reducing emulsifier ratio for HLB determination.

TABLE I.—RESULTS OF STUDIES ON THE DETERMINATION OF THE HLB OF ACACIA

Acacia Lot No.	HLB of Acacia When Used With Tween 20	Tween 40	Tween 60	Tween 80	Average HLB
Ruger, lot 38	11.87	11.81	11.83	11.84	11.84
Penick, lot WRB-52	11.87	11.90	11.92	11.84	11.88
Penick, lot WOB-1524	11.87	11.81	11.92	11.92	11.88
Average HLB	11.87	11.84	11.89	11.87	11.87

TABLE II.—THE EFFECT OF METHOD OF AGITATION ON THE HLB OF ACACIA

Acacia Lot No.	HLB of Acacia By Waring Blendor	Hand-Shaking
Ruger, lot 38	11.87	11.84
Penick, lot WRB-52	11.84	11.92
Penick, lot WOB-1524	11.92	11.87
Average HLB	11.88	11.88

Table I that there is no significant influence of the lot or source of the acacia on the HLB value. It should be pointed out, however, that only three different lots of acacia were available for this study and therefore it does not necessarily imply that all acacia samples would show an average HLB of 11.87. It is a well-known fact that some samples of acacia (tears *vs.* powder, for example) show some different physical properties. It would not be surprising that there might be some slight difference in HLB values with different lots of acacia.

In order to determine if method of agitation had any influence on the HLB of acacia, emulsions were prepared as previously outlined, but were agitated in one series by stirring two minutes in a Waring Blendor at the specified intervals. A second series was agitated by shaking by hand for two minutes at the specified intervals. In this determination, all three lots of acacia were used but only Tween 80 was used as the surfactant since it appeared that there was no significant difference in the effect of surfactant. The results are listed in Table II.

It appears from these results that method of agitation has no influence on the HLB of acacia so long as the degree of agitation is essentially uniform for each series and sufficiently violent to form an emulsion of fairly small globules. It should be emphasized that emulsions made on the Waring Blendor were finer than those made by hand and that the degree of creaming was also considerably less. However, the end result was that the HLB was unchanged.

CONCLUSIONS

1. A study of the effects of various surfactants on the HLB of acacia has been conducted and it was found that the Tween used had no effect on the HLB.

2. An investigation of the differences in lots on the HLB of acacia revealed that no change in HLB was caused when three different lots of acacia were used.

3. The study indicates that the method of agitation had no effect on HLB values as long as the agitation was uniform and sufficient to form a stable emulsion.

4. An HLB value for acacia which is more indicative of its ability to emulsify liquid petrolatum has been determined. The HLB value for acacia determined in this study was found to be 11.87.

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Pharmacological Investigation of Certain *Valeriana officinalis* L. Extracts

By J. A. ROSECRANS, J. J. DEFEO, and H. W. YOUNGKEN, Jr.

The roots of *Valeriana officinalis* were extracted by various solvent extraction procedures and the fractions prepared were tested for their biological activity in an attempt to isolate the most active constituents. Two fractions, V103 and V115, both showed some ability to potentiate the pentobarbital sleeping time in rats. The V115 fraction was also shown to possess greater hypotensive properties than V103. Countercurrent distribution extraction of V115 yielded three fractions, one of which produced hypertensive effects and the other two produced hypotensive effects. Due to inability to reproduce another active V115 fraction, V103 was selected for further testing. The V103 fraction depressed the general activity of rats without producing sleep, exhibited respiratory depressant properties, and produced hypotensive effects in rats, rabbits, cats, and dogs. V103 appears to produce hypotension partly through parasympathomimetic activity and blockage of the carotid sinus reflex. It inhibited the activity of isolated intestinal tissue at low doses and produced a secondary stimulant effect at higher doses which could be blocked by atropine. V103 is relatively nontoxic.

THE DRIED ROOTS of *Valeriana officinalis* L., a perennial herb indigenous to Europe and Asia, have been employed for sedative purposes in various forms since early times (1–3). The drug has been described in the United States Pharmacopeia, 1920–1940, and the National Formulary, 1940–1950, and various investigators have reported the alcoholic tinctures of the root drug to be useful in the treatment of hysteria, some types of insomnia, and as analgesics.

Chevalier (4) showed that valerian root produced depressant and paralyzing effects on the central nervous system and concluded that this action was due to a volatile alkaloidal constituent. Valerian's sedative properties were further investigated by Boruttau (5) who showed that it potentiated barbiturate-induced sleep in man, but not in lower animals. The sedative properties were discussed by others who concluded that valerian was a useful sedative, especially in cases of hysteria (6–8).

Several other pharmacological actions have been described for this plant drug. Pilcher (9) demonstrated that the fluidextract of valerian depressed uterine activity. It was also shown that the infusion of valerian and sodium valerate were inactive. Valerian was also shown to possess one fiftieth the potency of cocaine as an anesthetic (10). Blackie and Ritchie (11) also found that valerian possessed hypotensive properties. They demonstrated that atropine blocked this effect by fifty per cent. Bejrablaya (12) reinvestigated valerian's sedative properties. The results indicated that alcoholic extracts of the dried roots had some ability to potentiate barbiturate sleeping time in animals, a property common to several tranquilizers (13).

In the course of a phytochemical examination of the valerian root by our Department of Pharmacognosy several extractives and their isolates were prepared by conventional solvent extraction procedures, including the use of countercurrent distribution methods. This report describes certain pharmacological properties observed from the study of the most active extract, a 90 per cent ethyl alcohol extract of the crude valerian root and of two fractions prepared from this extract.

METHODS

Two kilograms of dried and powdered number 40-mesh valerian root[1] was extracted in a continuous extraction chamber with ether (b.p. 380–480°) until completely exhausted. Approximately 3 Kg. of the defatted drug was then further extracted by 90% ethyl alcohol in a Soxhlet apparatus. The ethyl alcohol-soluble extract (211 Gm.) was designated V103 and then set aside for testing.

Aliquots of the alcohol extract (V103) were then refluxed for six hours with a mixture (4:1) of methyl alcohol and concentrated hydrochloric acid. The soluble portion was filtered and the solution shaken with distilled water. Insoluble materials were filtered off and the aqueous extract subjected to further fractional extraction using U. S. P. ether. The aqueous phase was then concentrated and extrated with n-butyl alcohol, whereupon the water-soluble portion was separated, evaporated to dryness, and re-extracted with methyl alcohol. The methyl alcohol extract was refluxed with activated charcoal for two hours, filtered, and set aside for further testing. This extract was designated V115 and approximately 4 Gm. was obtained in each case.

[1] S. B. Penick Co., 50 Church Street, New York, N. Y.

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Aliquots of V115, 2 to 5 Gm., were mixed in a post countercurrent distribution apparatus with ethyl acetate as the moving phase and water as the stationary phase. Following each cycle of extraction, tubes number 1–50 were tested. Of these, tubes number 2 (40%), 4 (30%), and 6–11 (combined 15%) showed activity during preliminary pharmacological testing. These were therefore set aside for more thorough evaluation.

Alcoholic extracts of valerian were first screened for their effects on the pentobarbital sleeping time in rats (14) and for their hypotensive effects in rats. The alcohol extract (V103) was further evaluated as to its general pharmacological properties and its mechanism of action on blood pressure.

Pentobarbital Sleeping Time in Rats.—Each experiment utilized twenty male albino rats (Wistar strain) which were starved twenty-four hours prior to the experiment. Ten animals were used in each control and experimental group. The valerian extracts in each case were concentrated to dryness, dissolved in distilled water, and administered intraperitoneally thirty minutes prior to an anesthetic dose of pentobarbital sodium (35 mg./Kg.). The duration of sleeping time was recorded from the time the animals lost their righting reflex until the time of recovery.

Hypotensive Studies.—The experiments were conducted in rats (150–225 Gm.), cats (2.3–3.0 Kg.), dogs (14–19 Kg.), and rabbits (2.75–3.2 Kg.) of both sexes. All animals except the rats were anesthetized with an intraperitoneal injection of 25–35 mg./Kg. pentobarbital sodium. The rats were anesthetized with an intraperitoneal injection of 1.2 Gm./Kg. urethane because of its lack of effect on blood pressure. The effects of the extracts on blood pressure were recorded by direct cannulation of either the carotid or femoral artery with a system consisting of mercury manometer connected to the cannula by a fluid bridge of 7.5% sodium citrate solution. The effects on the blood pressure were recorded on a slowly moving smoked kymograph. Heparin sodium, 0.01 mg./Kg. was injected intravenously, and an equal amount added to the cannula to prevent clotting. Extracts in a dry form were dissolved in distilled water and administered via the femoral vein.

The indexes of activity used to compare different fractions possessing a hypotensive effect are as follows: (*a*) the maximum percentage drop of the blood pressure caused by an active fraction (40–50%), (*b*) the duration of hypotensive effect.

In attempting to determine the mechanism of hypotension of the alcoholic extract (V103) several experiments were conducted in dogs and cats. The effects of the drug on the carotid sinus reflex were tested by bilateral carotid occlusion for thirty seconds prior to, during, and following the hypotensive effects of the alcohol extract of valerian. Epinephrine (0.025 cc. of 1:1000) was administered prior to and during the hypotensive effects of the drug. The pre- and postganglionic fibers of the superior cervical ganglia innervating the nictitating membrane were also isolated, and stimulated electrically prior to and during the hypotensive effects of the drug. In three animals atropine (1 mg./Kg.) was administered prior to a second dose of valerian. Total vagotomy was performed before a second injection of valerian in two rats.

Intestinal Motility.—A 1-cm. segment of rabbit ileum was resected and placed in a muscle bath containing 70 cc. of oxygenated Locke-Ringer's solution maintained at 37°. Rhythmical contractions of the muscle were recorded on a slowly moving smoked kymograph via a pulley system. The alcohol extract of valerian was added to the bath with a 1-cc. syringe and allowed to remain in the bath for a period no longer than five minutes.

Short Term Toxicity Study.—Two groups of albino rats were employed in these studies. The first group consisted of eleven male rats (125–150 Gm.). This experiment terminated at the end of forty-five days during which the alcohol extract was administered intraperitoneally daily in doses ranging from 400–600 mg./Kg. At weekly intervals blood and urine analyses were conducted. Erythrocyte and leukocyte counts and clotting times were determined by the usual methods (15). Urine albumin and dextrose, urine pH, and specific gravity were likewise also determined.

A second group of twelve albino male and female rats weighing 40 to 60 Gm. also received valerian intraperitoneally daily in doses ranging from 400–600 mg./Kg. This experiment was performed to determine the effect of valerian on the weights of growing rats.

LD_{50} Determination.—Fifty male and female mice of the C57 strain were utilized. The mice were starved twenty-four hours prior to the experiment and were divided into five groups of ten each. Each group consisting of six female and four male mice was administered the same dosage ranging from 0.9–4.0 Gm./Kg. The doses were administered intraperitoneally and mortality was recorded within twenty-four hours following administration of the drug. The results were analyzed by the Litchfield and Wilcoxon (16) method.

General Activity.—In a preliminary experiment twelve mice (22–30 Gm.) and twenty rats (75–125 Gm.) of both sexes were administered the alcohol extract intraperitoneally and observed for a period of several hours. The drug was administered in doses ranging from 0.3 to 6.0 Gm./Kg. Effects were recorded as a change from the normal activity of the animal.

A second experiment utilizing activity cages was conducted. Spontaneous movements of male mice (22–30 Gm., strain C56) were recorded on a slowly moving smoked kymograph via a pulley system attached to a cage which was sensitive to vertical and horizontal movements. The activity of two groups of six mice each were recorded simultaneously in two different cages, with one group acting as a control. The alcohol extract of valerian was administered intraperitoneally in doses ranging from 300 to 900 mg./Kg. thirty minutes prior to beginning the experiment. The control mice received an equal volume of normal saline. The activity of the control and experimental groups was calculated as the total number of movements per minute every ten minutes for one hour.

RESULTS

Bejrablaya (12) reported that several fractions prepared from valerian potentiated the pentobarbital sleeping time in rabbits. Besides the implication of a tranquilizing or sedative action of vale-

rian, it was felt that this procedure could yield an excellent bioassay procedure in attempting to isolate an active fraction of valerian. However, in twenty-five experiments utilizing this procedure, only five experiments indicated any statistical significance, and it could only be concluded that the V103 and V115 fractions had effects on the sleeping time of anesthetized rats (Fig. 1).

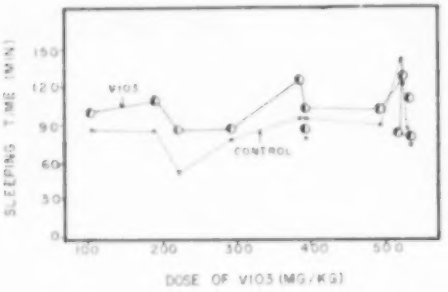

Fig. 1a.—Effects of the V103 fraction of valerian on the average pentobarbital sleeping time in rats.

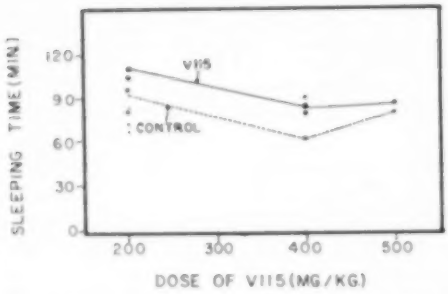

Fig. 1b.—Effects of the V115 fraction of valerian on the average pentobarbital sleeping time in rats.

The failure of this method led to the use of a hypotensive test in rats. Two fractions, V103 and V115, proved to have hypotensive properties. The V115 fraction, because of its greater potency, was then extracted by a countercurrent distribution method as previously described. This procedure yielded three fractions which acted differently on the blood pressure of rats. Two fractions (tube 2 and the combined 6–11 tubes) produced hypotensive effects, one with a delayed onset of action, (the combined tubes 6–11) and the third (tube 4) produced a hypertensive effect (Fig. 2). In attempting to duplicate these results two other countercurrent extractions were performed, but only the hypertensive fraction was obtained. By comparing the residue of the three countercurrent extractions to that of V115 it was apparent that with each succeeding extraction less of the hypotensive principle was extracted.

Following this difficulty in producing a second series of active V115 fractions and because of the greater potency of the alcohol extract (V103) (Fig. 3) it was subjected to further study with the following results:

The V103 fraction of valerian was found to depress the general activity of unanesthetized mice and rats. It depressed respiration as evidenced by the development of cyanosis in animals receiving large doses (3 Gm./Kg.) of the drug. An apparent sedative dose lies in a range from 0.6 to 1.2 Gm./Kg. as observed from the preliminary experiments on general activity. This sedative dose range was further verified utilizing activity of mice. At this dose, 600 mg./Kg., there was some evidence of a peripheral dilation and a moderate cyanosis as evidenced by the development of red color in the paws and ears of rats and mice.

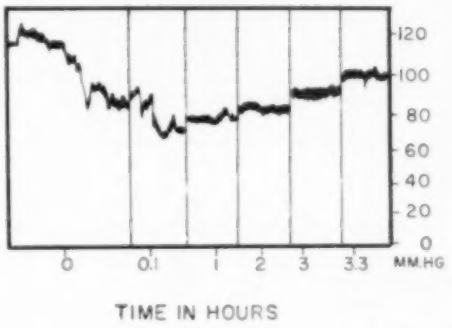

Fig. 2a.—The hypotensive effect of tube 2 in a rat. Tube 2 was obtained from the first countercurrent extraction of V115; dose, 30 mg./Kg.

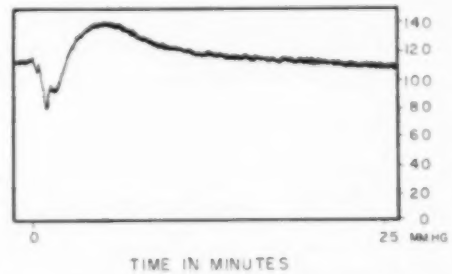

Fig. 2b.—The hypertensive effect of tube 4 in a rat. Tube 4 was obtained from the third countercurrent extraction of V115; dose, 200 mg./Kg.

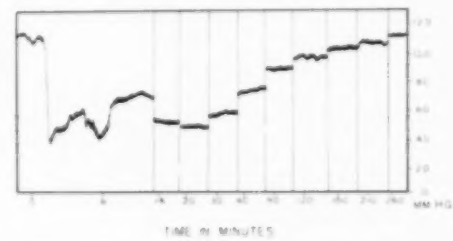

Fig. 3.—The hypotensive effect of the V103 fraction of valerian in a rat; dose of V103 500 mg./Kg.

The minimal lethal intraperitoneal dose was calculated to be about 3.0 Gm./Kg. At this dose the animals appeared to be in a state of complete paralysis since they were unable to right themselves when placed on their backs. It should also be mentioned that even though these animals could not right themselves, they did respond to external stimuli and did not appear to be asleep. Death in rats and mice ensued at a dose of 6 Gm./Kg. in about ten minutes with death sometimes being preceded by clonic-tonic convulsions.

The V103 extract produced hypotensive effects in rats, rabbits, cats, and dogs, but it was difficult to demonstrate a proportional dose-response relationship due to respiratory depression.

In cats and dogs, V103 blocked the carotid sinus reflex in all experiments (Fig. 4). The drug had no blocking effect on epinephrine, and even appeared to potentiate its activity. Atropine, however, did block valerian's hypotensive effects by 50% as was also pointed out by Blackie and Ritchie (11). In two experiments with rats, total vagotomy blocked the effects of valerian equal to the same degree produced by atropine. Valerian did not block the superior cervical ganglia as evidenced by the lack of an effect on the nictitating membrane preparation of the cat.

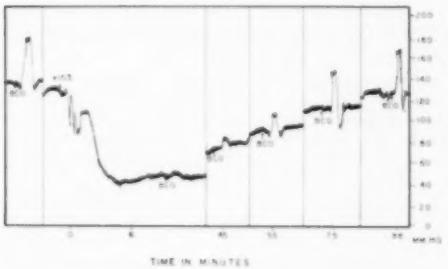

Fig. 4.—The hypotensive effect of the V103 fraction of valerian (84 mg./Kg.) in the dog with special reference to the carotid sinus reflex BCO = bilateral carotid occlusion.

It thus appears that the valerian root extract does not produce its hypotensive action by either ganglionic or sympathetic blockade. However, there is evidence that valerian possesses a parasympathetic action mediated through vagal stimulation which can explain part of its hypotensive activity. It can further be added that since valerian extract produced hypotension only by partial parasympathetic action, its central nervous system depressant activity could add to the hypotension. Further evidence that valerian could produce its hypotensive effects through central nervous system depression was reported by Dukrey and Kohler (17). They showed that valerian still possessed sedative activity after decerebration, thus placing the site of central nervous system depression at some area lower than the cortex, possibly the medulla, which is also the site of the vasoconstrictor center. It is possible that this central effect could in part explain the effects on the carotid sinus reflex.

Valerian (V103) also has the ability to relax smooth muscle in very low concentrations (0.0071%), but as the dose is increased to 0.43% a secondary excitant effect which can be blocked by atropine was observed. It is possible that this excitant effect is also related to the parasympathetic action referred to previously.

V103 is relatively nontoxic as evidenced by its failure to produce any significant change in the weight, blood, and urine of rats. There was some evidence that valerian produced some antidiuretic effects as shown by the decreased urinary excretion as compared with the per cent urinary excretion following the termination of the experiment. The high specific gravity of the urine during the testing period as compared to the control animals may also indicate an antidiuretic effect in rats.

The relatively high LD_{50} dose, in mice, 3.3 Gm./Kg. (2.53–4.29) indicates V103 has a low toxicity.

SUMMARY

A study was made of certain pharmacological actions produced by extracts of *Valeriana officinalis* L. roots. An alcoholic extract, V103, and a fraction separated from it, V115, were found to potentiate the pentobarbital sleeping time in rats. Both also produced hypotensive effects in rats. The V115 fraction showed greater hypotensive activity.

Three fractions which were extracted from V115 through countercurrent extraction exhibited different effects on the blood pressure of rats. Two fractions exhibited hypotensive properties, and the third exhibited hypertensive properties.

The alcoholic extract, V103, also produced hypotensive effects in rabbits, cats, and dogs. Its hypotensive effects appeared to be due to a combination of parasympathetic activity, carotid sinus blockade, and probably through central nervous system depression action. The latter action was also implied by the work of Dukrey and Kohler (17). V103 was further shown to lack any effect on the autonomic ganglia and on sympathetic fiber activity. It produced a depression of the central nervous system without producing sleep and showed evidence of causing respiratory depression in higher dosage.

The alcoholic extract relaxed smooth muscle in low concentrations, but at higher concentrations produced secondary excitant effects which can be blocked by atropine. Some antidiuretic effects were also observed. The LD_{50} dose (3.3 Gm./Kg.) of the extract in rats was considered rather high, thus indicating a low order of toxicity.

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Biological Disposition of Apomorphine II

Urinary Excretion and Organ Distribution of Apomorphine

By P. N. KAUL†, E. BROCHMANN-HANSSEN, and E. LEONG WAY

A method for estimation of free and "bound" apomorphine in biologic media is described. Apomorphine, after parenteral administration to different animal species, is biotransformed and excreted in urine largely in its "bound" form. Studies on the organ-distribution of injected apomorphine in rats revealed a localization of "bound" apomorphine in the liver.

APOMORPHINE is well known in chemical literature, but little is known about its fate in the animal body. Valenti (1), and later Eggleton and Hatcher (2), failed to detect apomorphine in the urine of dog and rabbit after its administration. However, Veit (3), reported that apomorphine administered to monkeys and cats could be detected in the brain tissue within one to two hours.

Bamford (4) conducted experiments with rabbits to show that no apomorphine appeared in the stomach contents after subcutaneous injection, even when it was administered in large doses. Various investigators (5–7) reported injection experiments in which saliva and urine samples from horses were tested for the presence of apomorphine. The authors could not detect the alkaloid by ultraviolet spectrophotometry or by several color reactions.

This paper describes the urinary excretion of apomorphine in rabbits, rats, and horses, and the tissue distribution of the drug in rats.

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The paper appearing in THIS JOURNAL, **48**, 638(1959) may be regarded as part I of this series.
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† Present address: Department of Pharmacology, University of Melbourne, Carlton N. 3, Victoria, Australia.

EXPERIMENTAL

Estimation of Apomorphine.—The procedure of Kaul, *et al.* (8), was modified for routine determinations in biological materials, the major changes being reduction in organic solvent volume and adoption of single extraction techniques. Solvents were measured with fixed-volume stop-syringes.

For determination of free apomorphine, 10 ml. of urine or tissue homogenate (1 Gm. diluted to 10 ml.) in a 50-ml. glass-stoppered centrifuge tube was neutralized with solid sodium bicarbonate to pH 7 ± 0.5 and extracted with 27.5 ml. of ethyl acetate for two minutes. A 25-ml. aliquot of the organic layer was removed after centrifugation and shaken for two minutes with 5.5 ml. of 0.05 *N* hydrochloric acid. The mixture was centrifuged, and a 4-ml. aliquot of the acid layer was pipetted into another tube and analyzed by the method previously described (8), 4 ml., instead of 10 ml., of isoamyl acetate being used for the final extraction.

Since the alkaloid was found to be excreted in a conjugated ("bound") form, it was necessary to liberate apomorphine by hydrolysis prior to its

estimation. The optimum conditions for the hydrolysis of "bound" apomorphine were studied in samples of postadministration urine, the acid strength and the heating time at reflux temperature being varied successively. Optimum hydrolysis of "bound" apomorphine occurred when the samples were refluxed for twenty minutes with 4 *N* hydrochloric acid (Fig. 1). Authentic apomorphine, on treatment under these conditions, showed a decomposition of 10 ± 2%.

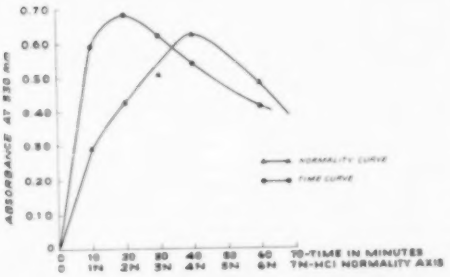

Fig. 1.—Effects of acid strength and time of refluxing on the hydrolysis of "bound" apomorphine.

For determination of total apomorphine (free and "bound"), 5 ml. of the biologic fluid was refluxed with 4 ml. of 9 *N* hydrochloric acid for twenty minutes. After cooling, the solution was quantitatively transferred to a 50-ml. glass-stoppered centrifuge tube. Sodium bicarbonate was added to adjust the pH to 7 ± 0.5, and the alkaloid was estimated as described for free apomorphine. A concentration of 1 mcg./ml. of apomorphine, when carried through this procedure, yielded an absorbance of 0.092.

Blanks for the determination of free apomorphine were found to be less than 0.005 absorbance units for urine and tissue homogenates. Blanks for total apomorphine analyses were less than 0.03 units for tissue homogenates and less than 0.10 for urine. A correction for urine blank was made by subtracting the value obtained on a sample of urine collected from the same animal prior to apomorphine administration. Subsequent studies indicated that blanks can be substantially reduced by washing the acid hydrolysate of urine with two 5-ml. portions of ethyl acetate at pH 1.

Recoveries of added apomorphine were 80 ± 3% for urine and in excess of 90% for the homogenates. The basis for low recoveries from urine has been discussed (8).

Appraisal of the Method.—In addition to the specificity tests reported previously (8), the specificity of the analytical method was also confirmed on the basis of the partition behavior of apomorphine recovered from biological material.

A sample of postadministration horse urine was hydrolyzed, neutralized to pH 7 ± 0.5, and extracted with ethyl acetate. Ten-milliliter aliquots of the ethyl acetate extract were shaken for one minute with 10-ml. portions of McIlvaine buffer solutions of pH 4, 5, and 6 and with 0.05 *N* hydrochloric acid. The same procedure was repeated with preadministration horse urine to which authentic apomorphine hydrochloride had been added. The estimation of apomorphine in the buffer and the acid extracts were made by the modified method described above. Amounts extracted by 0.05 *N* hydrochloric acid were taken as 100%. Table I shows that there is a close agreement between the percentages of apomorphine extracted by buffers of different pH values from the postadministration urine and from urine containing authentic apomorphine. This indicates that the method estimates apomorphine with a high degree of specificity.

Table I.—Extraction at Different pH Values of Authentic Apomorphine and of Apomorphine Recovered from Urine after Administration of the Drug

pH of Extracting Solution	Per Cent Extracted	
	Authentic Apomorphine	Postadmin. Apomorphine
1.8 (0.05 *N* HCl)	100.0	100.0
4.0 Buffer	96.7	94.4
5.0 Buffer	67.6	62.4
6.0 Buffer	15.4	17.9

Additional evidence that the substance liberated by acid hydrolysis is apomorphine was confirmed by various qualitative tests. The alkaloid liberated on hydrolysis, when isolated into 0.1 *N* hydrochloric acid, exhibited an ultraviolet absorption curve typical of apomorphine and gave the following identity tests for apomorphine: (*a*) A portion of the acid solution produced a red color when treated with a few drops of concentrated nitric acid (9). (*b*) A pink color was produced when a mixture consisting of 5 ml. of the acid solution, 2 drops of 0.01 *M* iodine, and 0.5 Gm. of sodium bicarbonate was shaken with 10 ml. of ether (10). (*c*) A portion of the acid solution turned green when heated with mercuric chloride at pH 6. An isoamyl acetate extract of this green solution showed an absorption spectrum identical with that of authentic apomorphine subjected to the same treatment (8). (*d*) Another portion of the acid extract was evaporated to dryness under reduced pressure, and the residue was dissolved in 0.5 ml. of ethanol. The solution was chromatographed on paper alongside authentic apomorphine hydrochloride, the solvent system used being butanol, acetic acid, and water (4:1:5). Both spots had the same R_f value and exhibited a similar bluish-green fluorescence under ultraviolet light.

Urinary Excretion of Free Apomorphine.—Rabbits and rats were given 10 mg./Kg. of apomorphine hydrochloride intraperitoneally. Urine samples voided over twenty-four hours were collected over acid and analyzed for free apomorphine. No free drug was found in the urine of several rabbits, whereas two rats excreted, respectively, 3.8 and 4.0% of the given dose as the free alkaloid. The pH of rabbit urine was 8 ± 0.5 and that of rat urine 6 ± 0.5. Since the pH of freshly voided rabbit urine is usually basic, and apomorphine is known to be unstable under alkaline conditions, a study of the effect of pH on the stability of apomorphine at body temperature was made. Table II shows that the rate of decomposition of apomorphine increases rapidly with an increase of pH.

TABLE II.—EFFECT OF pH ON STABILITY OF APOMORPHINE AT 37°

pH of Buffer Soln. (McIlvaine)	% Recoveries at Various Time Intervals, hr.				
	0*	2	4	6	8
5.8	100.0	98.0	93.6	89.3	85.4
7.0	100.0	92.6	77.7	67.8	54.2
7.9	100.0	71.2	46.3	29.5	18.4

* Recoveries at zero time were taken to be 100% and the recoveries at the other time intervals calculated on this basis.

Studies on the stability of apomorphine in urine yielded results consistent with the above findings. When the alkaloid was incubated at 37 ± 1° with normal rabbit urine of pH 7.6, the recovery was 23.9% after twelve hours. Therefore, one might expect that any free apomorphine excreted in the urine would gradually decompose when exposed to the alkaline conditions in the bladder. This effect could be minimized by systemic acidification of urine. Thus, 1 Gm. of ammonium chloride in 100 ml. of water given to a rabbit through a stomach tube, reduced the pH of the urine from 7.6 to 5.4, and incubation of apomorphine at 37° with this urine resulted in a recovery of 87% after twelve hours. When the excretion of apomorphine was studied in two rabbits receiving ammonium chloride prior to the injection of apomorphine, about 3% of the dose was recovered in the free form. It should be noted that no free apomorphine was found in the urine of horses (Table III) and that horses generally excrete an alkaline urine.

Excretion of "Bound" Apomorphine.—The amount of total apomorphine excreted in urine over a twenty-four hour period was determined for six rabbits and six rats receiving a 10 mg./Kg. dose intraperitoneally. The mean excretion of total apomorphine in rabbits was found to be 49.9% with a range of 43 to 57%; in rats 46.2% with a range from 40 to 55%. Since rabbits do not excrete any free drug, the total apomorphine represents "bound" apomorphine. In the case of the rat, about 96% of the total apomorphine excreted is in the form of "bound" apomorphine.

The excretion of apomorphine was also studied at selected time intervals in rabbit, rat, and horse. Two animals of this latter species were given 75 mg. of apomorphine subcutaneously and urine specimens were collected by catheterization.

Table III shows that the rabbit excretes the major fraction of apomorphine in the bound form within twenty-four hours. However, measurable amounts are still excreted for as long as seventy-two hours. Similar findings were noted in the rat. In the horse, the recovery was less than in the rabbit and rat. The dosage administered was also smaller, being less than 0.2 mg./Kg. However, this dose was sufficient to produce pharmacologic effects lasting about two hours. Observations[1] made on horse No. 1 indicated that the animal was nervous, excitable, sweated profusely, and appeared to have sharpened reflexes. Temperature increased from 37.6° to 39.2°, pulse from 36 to 60, and respiration from 9 to 18. Free apomorphine was not detectable in either of the two horses, hence, the total apomorphine represents essentially "bound" apomorphine. From 14 to 26% of the dose was accounted for within six to eight hours, after which the levels of "bound" apomorphine became too low for detection.

Organ Distribution.—Three Long Evans rats, weighing approximately 200 Gm., were used to study the distribution of apomorphine hydrochloride in various organs and tissues of the body. An intravenous dose of 2 mg. of the drug was administered to each animal. After fixed time intervals the organs were removed, homogenized in 1 *N* hydrochloric acid, and analyzed for free and "bound" apomorphine.

At none of the time intervals (fifteen, sixty, and eighty minutes) could free apomorphine be detected in any of the organs and tissues analyzed (blood, liver, lungs, kidney, brain, and skeletal muscles), the concentration being less than 4 mcg./Gm. of tissue. However, "bound" apomorphine was found in appreciable quantities in the liver but not in other organs. The concentration of "bound" apomorphine in the liver was highest (43 mcg./Gm. of tissue) at fifteen minutes after injection. At sixty minutes the level was 23 mcg./Gm. of the tissue, and by eighty minutes it had decreased to 11 mcg./Gm. At the latter time interval about one-fourth of the injected dose was found as "bound" apomorphine in the gastrointestinal tract.

DISCUSSION

The present studies indicate that no free apomorphine is excreted in the urine of rabbits and horses. The pH of rabbit urine was usually 8 ± 0.5. However, when systemic acidosis was induced by the oral administration of ammonium chloride and the pH of urine was reduced to 6 ± 0.6, about 3% of the dose of apomorphine was excreted in the unchanged form. It appears, therefore, that the excretion of a small percentage of the free drug depends on the pH of urine. This may be due to stabilization of the excreted free apomorphine by acid pH. Moreover, such a pH would also favor ionization of the alkaloid. This would promote its excretion in the urine, presumably by decreasing tubular reabsorption of the drug. The increased excretion of some organic bases as a result of acidification of urine is well known (11, 12, 13). Supporting this view was the finding that rats with urine of pH 6 ± 0.5, excreted about 4% of the given dose as free apomorphine.

Authentic apomorphine, on treatment under the conditions used for hydrolysis of the "bound" material, showed a decomposition of 10 ± 2%. This means that the true values of the "bound" apomorphine are actually higher than those reported in this paper, because some decomposition of the liberated apomorphine must have occurred during the hydrolysis.

Veit (3) reported that he found about 30% of the dose in the brain of monkeys seventy minutes after administration of apomorphine. However, the present studies on organ distribution in rats indicated only insignificant levels (less than 4 mcg./Gm. or less than 0.2%) of the drug in the brain at fifteen and sixty minutes after intravenous injection. It appears unlikely that such a dis-

[1] We are grateful to Mr. George Jaggard, Director of Dalare Associates, for making the pharmacologic observations on the horse and for furnishing us with the horse urine specimens.

TABLE III.—URINARY EXCRETION OF APOMORPHINE IN THE RABBIT, RAT, AND HORSE

Species	Dose, mg./Kg.	Time of Collection, hr.	—% Excreted— Free	Bound	Total % Accounted For
Rabbit	10 mg./Kg.	24	0	51.2	...
		36	0	9.0	...
		44	0	8.8	...
		72	0	2.9	...
		90	0	0	71.9
Rat No. 1	10 mg./Kg.	24	4.0	44.0	...
		46	0	9.5	...
		54	0	3.2	60.7
Rat No. 2	10 mg./Kg.	24	3.8	51.5	...
		46	0	8.8	...
		54	0	4.0	68.1
Horse No. 1 410 Kg. gelding	75 mg. total	1.5	0	7.7	...
		2.5	0	11.5	...
		4.5	0	6.9	...
		8.0	0	trace	...
		24.0	0	0	26.1
Horse No. 2 390 Kg. mare	75 mg. total	1.25	0	3.5	...
		3.5	0	8.3	...
		6.0	0	1.8	13.6

crepancy can be explained in terms of differences in animal species.

The possibility that some of the "bound" apomorphine is excreted through the bile into the gastrointestinal tract is suggested by the fact that appreciable amounts of "bound" alkaloid were found in the liver and gut after parenteral administration of apomorphine to rats. Since "bound" apomorphine is finally excreted in urine, any amount transported via the bile would have to be reabsorbed in the gut and then eliminated via the kidney. This would explain the prolonged urinary excretion of "bound" apomorphine observed in rabbits.

SUMMARY

Apomorphine, after injection into animals, is excreted in the urine largely as "bound" alkaloid which, on hydrolysis, yields the free compound. Excretion of a small percentage of unchanged drug appears to be dependent on the pH of the urine. Normal rabbits and horses excrete little or no free apomorphine, whereas rats excrete about 4% of the dose in the free form.

Organ-distribution studies of apomorphine after intravenous injection into rats showed no detectable levels of the alkaloid in the central nervous system, but a high concentration of the "bound" alkaloid in the liver.

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Biological Disposition of Apomorphine III

Kinetics of Metabolism of Apomorphine and Role of the Liver in Its Biotransformation

By P. N. KAUL†, E. BROCHMANN-HANSSEN, and E. LEONG WAY

Studies on the rate of metabolism of apomorphine in intact mice revealed that the disappearance of injected drug and the appearance of "bound" apomorphine follow first-order kinetics, with the rate constants 8.15×10^{-2} min.$^{-1}$ and 6.07×10^{-2} min.$^{-1}$, respectively. Liver perfusion data and experiments on partially hepatectomized rats indicate that this organ is the main site of the metabolism of apomorphine.

PREVIOUS PAPERS in this series have described a method for quantitative determination of apomorphine (1) and subsequent application of this method in studying the fate and organ distribution of injected apomorphine (2). In this paper, the kinetics of metabolism and the role of liver in the biotransformation of the drug are described.

EXPERIMENTAL

Kinetics of Metabolism.—The rate of metabolism of apomorphine was studied in intact mice. Apomorphine hydrochloride, 50 mg./Kg., was injected into the tail vein of Albino mice (Webster strain) weighing 20 ± 4 Gm. (This dose killed none of five animals in preliminary trials, whereas 60 mg./Kg. and higher doses killed 4/4 mice.) The drug was given in isotonic saline containing 2 mg./ml. At fixed time intervals, a mouse was homogenized in a Waring Blendor, 1 *N* hydrochloric acid being used to make 1:10 w/v dilution of the mouse. Ten-milliliter and five-milliliter aliquots of the homogenate were assayed for free and total apomorphine, respectively, by the method described previously (2).

For blank values several untreated mice were carried through the procedure. The blank absorbance values for free and total alkaloid were less than 0.005 and 0.03, respectively. The recoveries of the drug added to mouse homogenates were within the experimental error of ±10%. Table I shows the per cent of the injected dose found as free and "bound" apomorphine at different time intervals. As is evident in Fig. 1, the disappearance of the injected apomorphine is virtually paralleled by the appearance of "bound" apomorphine.

When the data are plotted on a semilogarithmic scale, a straight line is obtained for at least a ten-minute interval (Fig. 2). Over this period the disappearance of apomorphine appears to follow first-order kinetics with a $t_{1/2}$ of eight and one-half minutes and a rate constant, k, equal to 8.15×10^{-2} min.$^{-1}$. The points at twenty and forty minutes do not fall on the line due, probably, to a lag period required for mobilization of the distributed drug from tissue depots to the site of metabolism.

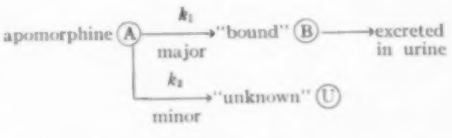
A theoretical first-order plot for the appearance of the "bound" apomorphine may be obtained by the relationship

$$B = A^0 \cdot f(1 - e^{-kt})$$

where B is the amount of "bound" formed at time t, A^0 is the amount of apomorphine at zero time, k is the first-order rate constant for the overall disappearance of apomorphine, and f is the total fraction of "bound" material formed at infinite time, which in this case is eighty minutes (Table I).

Figure 3 shows that the experimental values for the appearance of "bound" apomorphine approach the theoretical plot, indicating that the appearance of "bound" alkaloid also follows first-order kinetics.

From the data in Table I it appears that there is a loss of about 20% of total apomorphine after eighty minutes. This suggests that there is another minor pathway through which apomorphine disappears in the body. The metabolism of apomorphine may, therefore, be represented by a model shown below:



The overall disappearance rate constant k is the sum of k_1 and k_2. From this relationship and the equation, $f = k_1/k$, the values for k_1 and k_2 may be calculated as follows:

The total fraction, f, of "bound" apomorphine formed is 0.745 (Table I).

$$f = k_1/k = 0.745$$

$$\therefore k_1 = k \times 0.745 = 8.15 \times 10^{-2} \times 0.745 \text{ min.}^{-1} = 6.07 \times 10^{-2} \text{ min.}^{-1}$$

Since, $k = k_1 + k_2$,

$$k_2 = k - k_1 = 8.15 \times 10^{-2} - 6.07 \times 10^{-2} = 2.08 \times 10^{-2} \text{ min.}^{-1}$$

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† Present address: Department of Pharmacology, University of Melbourne, Victoria, Australia.

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TABLE I.—RATE OF METABOLISM OF APOMORPHINE IN INTACT MICE

Time, min.	Mice Studied	Per Cent of Initial Dose Found (T) Total	(F) Free	(T-F) "Bound"
0	2	100.0(99.5–100.5)	100.0(99.5–100.5)	0.0(−2.6 to 2)[a]
1	2	100.5(95–106)	93.6(92–95)	6.9(3–10)
5	4	99.9(94–105)	65.1(53–71)	34.8(28–41)
10	3	92.6(88–100)	45.8(37–52)	46.8(41–52)
20	4	91.3(87–98)	29.1(24–36)	62.2(61–64)
40	5	80.4(70–91)	10.2(4–20)	70.2(60–85)
80	5	79.7(76–84)	5.2(3–7)	74.5(68–79)

[a] The negative average value was taken as zero.

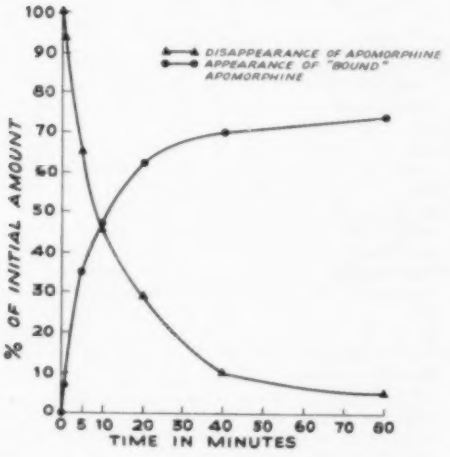

Fig. 1.—Disappearance of injected apomorphine and appearance of "bound" apomorphine.

Liver Perfusion.—The liver of a rat was cannulated and removed under anesthesia. The cannulated organ was placed in the glass circulation apparatus described by Brauer, *et al.* (3), and modified by Plaa (4). The perfusate entered the liver through the portal vein cannula and passed through the two hepatic veins into the thoracic *vena cava*. The perfusion fluid consisted of Ringer-Locke solution buffered to pH 7.5. The perfusate was not oxygenated, since preliminary experiments indicated that this treatment enhanced the decomposition of apomorphine. Bile was collected through polyethylene tubing inserted into the bile duct.

After ensuring the normal perfusion efficiency and the bile production of the liver, 1 mg. of apomorphine hydrochloride in 0.5 ml. of water was introduced into the perfusion circuit. Samples of the perfusate were removed at different time intervals and analyzed for free and "bound" apomorphine. At the end of forty minutes the liver was removed, homogenized, and assayed for the alkaloid. Under these conditions some decomposition of apomorphine occurs spontaneously; the recovery of apomorphine added in the same amount to Ringer-Locke solution was found to be only 80% at forty minutes. Consequently, a correction for the apomorphine perfusate value at each time interval was applied based on the apomorphine recovery from Ringer-Locke solution at the same interval.

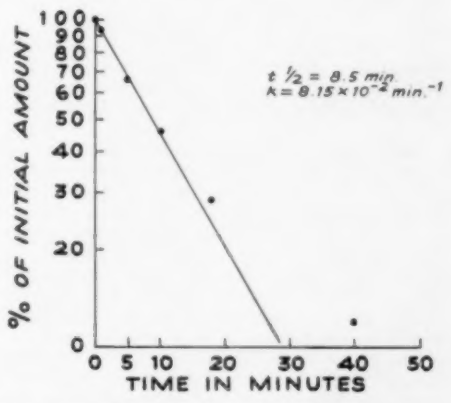

Fig. 2.—First-order plot for the disappearance of the injected apomorphine.

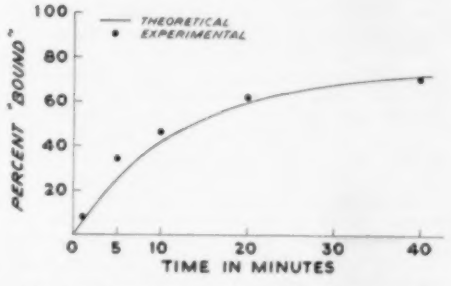

Fig. 3.—Theoretical and experimental cumulative first-order plot for the appearance of "bound" apomorphine.

TABLE II.—FATE OF APOMORPHINE IN ISOLATED PERFUSED RAT LIVER

Sample	Per Cent of the Injected Dose Free	"Bound"
2 min. Perfusate	10.0	0.0
10 min. Perfusate	2.0	20.7
40 min. Perfusate	5.3	26.7
40 min. Liver	13.4	40.0
Total accounted for after 40 min.	18.7	66.7

From the data in Table II it appears that apomorphine is very rapidly taken up by the liver as evidenced by its rapid disappearance from the perfusion fluid. It is then transformed into a "bound"

TABLE III.—EFFECT OF PARTIAL HEPATECTOMY IN THE TWENTY-FOUR-HOUR EXCRETION OF APOMORPHINE IN URINE OF RATS

	% Dose Excreted						
	Rat No. 1	2	3	4	5	6	Mean ± S. E.
Normal rats							
Free apomorphine	...	...	...	...	4.0	3.8	... ...
Bound apomorphine	...	...	...	...	44.0	51.7	... ...
Total apomorphine	40.0	46.0	46.1	42.0	48.0	55.5	46.3 ± 2.2
Hepatectomized rats							
Free apomorphine	...	...	...	2.2	1.6	2.2	... ...
Bound apomorphine	...	...	...	31.1	31.1	35.1	... ...
Total apomorphine	14.9	20.0	33.0	33.3	32.7	37.2	28.5 ± 3.6

form which is slowly released back into the perfusing fluid but appreciable quantities of both free and "bound" apomorphine remain sequestered in the liver.

Hepatectomy.—Experiments were performed to establish *in vivo* evidence that the liver is important for metabolizing apomorphine. Subtotal hepatectomy was performed on Long Evans rats (240 ± 40 Gm.) under ether anesthesia by removing the large median lobe and/or the left lateral lobe in the manner described by Higgins and Anderson (5). The animals were allowed twelve to sixteen hours to recover from the surgical procedure. Within this period regeneration of the liver occurs only to the extent of about 8 ±2% of the original weight of the organ. After the recovery period, a 2-mg. intraperitoneal dose of apomorphine hydrochloride was injected, and twenty-four-hour urine samples were collected and analyzed for the free and "bound" apomorphine. Similar samples were also collected from a group of normal rats receiving the same 2-mg. dose of the drug and analyzed in the same manner.

Table III shows that subtotal hepatectomy reduced the total apomorphine excreted in the twenty-four-hour urine. Normal animals excreted on the average 46% of the dose in twenty-four hours while the operated animals excreted only 29%. By application of the *t* test, it may be concluded that this disparity between the two groups is unlikely to be due to chance ($P < 0.01$). The decrease in total apomorphine excreted was primarily the result of decreased conjugation since only "bound" apomorphine excretion decreased. Since the free apomorphine excretion was not increased, the findings suggest that some pathway other than conjugation must be involved in the metabolism of apomorphine and that this route is not as dependent on the integrity of the liver. The fact, however, that the operated animals exhibited much more pronounced pharmacologic effects to apomorphine than normals, indicate that the liver is important for the detoxification of the apomorphine. In additional experiments, performed on younger rats weighing 120 ± 20 Gm., removal of about 70% of the liver resulted in an enhanced response to apomorphine. Three out of three animals died within one hour after receiving 2 mg. of the drug, whereas when the same dose was given to five normals of the same weight range, no deaths occurred.

Effect of Carbon Tetrachloride.—In order to establish additional *in vivo* evidence as to the role of the liver in the metabolism of apomorphine, the effects of this well-known hepatotoxin were studied on the rate of formation of "bound" apomorphine in mice. To produce liver damage, the carbon tetrachloride was administered at dosages several times the ED_{50} dose reported by Plaa (4). Carbon tetrachloride was diluted in peanut oil (0.18 ml. in 1 ml. oil) and injected subcutaneously. After twenty-four hours, the mice were given a 50 mg./Kg. dose of apomorphine hydrochloride intravenously. The animals were homogenized after twenty minutes, and aliquots of the homogenate samples were assayed for free and "bound" apomorphine. The results in Table IV show that carbon tetrachloride had little or no effect on the formation of "bound" apomorphine, since the amounts of free and "bound" apomorphine found in treated mice were within the range of those found in normal animals.

TABLE IV.—EFFECT OF CARBON TETRACHLORIDE ON THE METABOLISM OF APOMORPHINE IN MICE

No. of Mice	Dose of CCl_4 Given, mg./Kg.	Per Cent of Injected Dose Free	"Bound"
4	None (Normals)	29.1 (24–36)	62.2 (61–64)
1	1,000	30.5	62.6
1	3,000	24.3	62.8
1	4,000	29.8	51.3
1	5,320	29.0	59.1

The effect of carbon tetrachloride on the metabolism of apomorphine was also studied in rats by comparing the urinary excretion of the free and the "bound" alkaloid in the normal and the treated animals at various time intervals. Intraperitoneal doses (1,440 mg./Kg.) of carbon tetrachloride in peanut oil were given daily for three days during which the urinary excretion of apomorphine was studied. Table V gives the percentages of the alkaloid excreted by normal and treated rats. It can be readily seen that amounts of free and "bound" apomorphine excreted by the two normal and the two treated animals at all time intervals were quite similar, indicating that conjugation of apomorphine was not materially affected by treatment with carbon tetrachloride.

DISCUSSION

From the kinetic data of mice experiments, it appears that formation of "bound" apomorphine is the major pathway of metabolism. However, a gradual decrease of the total recoverable apomorphine (free and "bound") with time suggests the possible existence of minor pathway(s) through

TABLE V.—EFFECT OF CARBON TETRACHLORIDE ON THE METABOLISM OF APOMORPHINE IN RATS

Animals	Per Cent Apomorphine Found in Urine Collected						
	0–24 hr.		24–46 hr.		46–54 hr.		
	Free	Bound	Free	Bound	Free	Bound	Total
Normal	4.0	44.0	None	9.5	None	3.2	60.7
Normal	3.8	51.5	None	8.8	None	4.0	68.3
Treated	5.2	57.0	None	6.5	None	3.1	71.8
Treated	3.5	48.1	None	8.0	None	3.8	63.4

which about 20% of the drug is metabolized. There are several theoretical possibilities for this minor pathway. From the *in vitro* evidence that apomorphine oxidizes spontaneously at body pH in the presence of air, it would seem that the compound might, to some degree, be auto-oxidized in the body as well. No attempts were, however, made in this investigation to isolate such oxidation product(s).

Conjugation of apomorphine also appears to be a primary route for the detoxification of apomorphine in the rat, and the liver plays an important role in this regard. This is indicated by the facts that (*a*) the compound is rapidly taken up and bound by the perfused rat liver, (*b*) excretion of "bound" apomorphine in twenty-four-hour urine of the rat is considerably reduced after subtotal hepatectomy, (*c*) the surgical procedure also enhanced the sensitivity of the animals to apomorphine.

It is of interest that carbon tetrachloride did not appear to affect the conjugation of the alkaloid to any appreciable degree in the rat or the mouse. It has long been known that carbon tetrachloride causes damage of the liver. Plaa, *et al.* (4), have shown that the ED_{50} dose of carbon tetrachloride for producing prolongation of barbiturate sleeping time in mice is 154 mg./Kg. and that 480 mg./Kg. will invariably impair the bile production and the bromsulphalein extraction by the rat liver. These doses generally produce gross as well as microscopic liver damage. The dose of carbon tetrachloride used in our mice experiments was six to thirty-five times that used by Plaa to prolong pentobarbital sleeping time. It is quite possible that, even though many of the functional systems of the liver were deranged by the massive doses of carbon tetrachloride used, this treatment would not necessarily impair the ability of the organ to convert apomorphine into the "bound" form. That certain enzyme systems might show some resistance to carbon tetrachloride is supported by the findings of Christie and Judah (6) who reported that the oxidation of pyruvate, citrate, hydroxybutyrate, and malate was markedly reduced after intragastric administration of 4 Gm./Kg. of carbon tetrachloride to rats, whereas the oxidation by the liver of glutamate, ketoglutarate, choline, and succinate was normal.

It is of importance to consider the effects of carbon tetrachloride on conjugation mechanism since our preliminary evidence suggests that conjugation with glucuronic acid is involved in the formation of "bound" apomorphine. It is well-established that glucuronide conjugation occurs primarily in the liver and is a detoxification mechanism for phenolic compounds. The process involves an enzyme (glucuronyl transferase) in the microsomes which transfers glucuronic acid from uridine diphosphate glucuronic acid (UDPGA) to the phenolic acceptor (7). It has been recently reported that intraperitoneal injection of carbon tetrachloride reduces the hepatic glucuronide conjugating capacity of the guinea pig as measured by *p*-nitrophenol glucuronide formation *in vitro* (8). These results are difficult to reconcile with our findings *in vivo* that "bound" apomorphine in the mouse and the rat was not materially altered by treatment with carbon tetrachloride. It is possible that *p*-nitrophenol and apomorphine do not act as acceptors for a common microsomal enzyme, but to date no conclusive evidence for more than one glucuronyl transferase has been obtained. While considerations of species difference cannot be ignored, a more plausible answer may be that carbon tetrachloride may affect glucuronide conjugation mechanism to the extent that the effects can be detected *in vitro* but the damage is not of the magnitude which can be easily demonstrated *in vivo*, particularly since other organs such as the kidney and gastrointestinal tract are also known to contain glucuronyl transferase (9). In support of such an argument, it was reported (8) that although acute liver damage by carbon tetrachloride produced a significant reduction in the total hepatic glucuronide as measured *in vitro*, the glucuronyl transferase activity in the microsomes isolated from damaged liver tissue was not diminished. The authors suggested that the damage produced by carbon tetrachloride does not involve the microsomes of all liver cells and cited the studies of Myron (10) who also observed that liver damage produced by carbon tetrachloride was not uniform but that midzonal cells were more vulnerable than centrilobular cells. Further, it was noted in the same study that the activity of succinic dehydrogenase, another particulate enzyme, was not diminished in cells peripheral to midzonal area of the liver lobule. A more definitive explanation can be obtained only by further studies.

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Evaluation of Certain Psychopharmacological Compounds

By ANGELO R. FURGIUELE†, WILLIAM J. KINNARD, and JOSEPH P. BUCKLEY

The effects of chlorpromazine, pentobarbital, 5-(*o*-methoxyphenoxymethyl)-2-oxazolidone (OM-518), and 5-(*o*-methylphenoxymethyl)-2-oxazolidone (OM-520) on spontaneous and forced motor activity were investigated in male, albino, Swiss mice. Chlorpromazine produced approximately the same degree of depression of spontaneous activity and forced motor activity. Pentobarbital was mainly effective in depressing forced motor activity, that is, doses that produced minimal effects on spontaneous activity were highly effective in depressing forced motor activity. The two oxazolidone compounds produced marked depression of spontaneous activity in doses which did not alter forced motor activity.

KINNARD AND CARR (1) have described a method of differentiating between types of depressants utilizing photocell activity cages and the Rotarod (2). This method appeared to be especially useful in identifying compounds which produced depression of spontaneous activity of mice in doses which produced little or no effect on forced or coordinated motor activity. Several papers have recently been published or presented on the ataractic properties of oxazolidone compounds (3, 4). The purpose of this present study was to compare the effects of two new oxazolidone compounds, 5-(*o*-methoxyphenoxymethyl)-2-oxazolidone (OM-518) and 5-(*o*-methylphenoxymethyl)-2-oxazolidone (OM-520), (Fig. 1), with a cerebral depressant (pentobarbital sodium) and a potent phenothiazine ataractic agent (chlorpromazine).

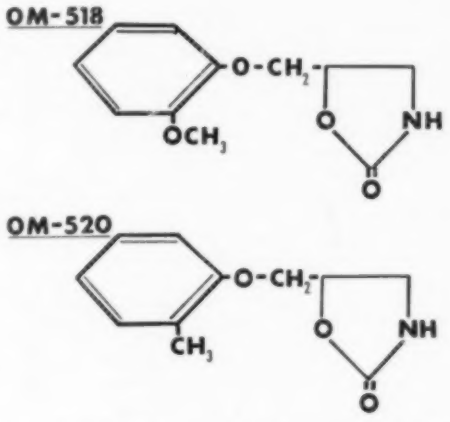

Fig. 1.—Chemical structure of OM-518 and OM-520.

EXPERIMENTAL

Effects on Spontaneous Motor Activity.—The effects of OM-518, OM-520, chlorpromazine, and sodium pentobarbital on spontaneous motor activity of male, albino, Swiss-Webster mice were studied using photocell activity cages. The cages were modified after those described by Kinnard and Carr (1) only in that the photocell units were increased from one to two and placed 90 degrees apart. The temperature of the cage room was maintained at 24 ± 1° and the room was illuminated at all times. Recording instruments were placed in another room to prevent the equipment noises from influencing the mice. Fresh solutions of the compounds or normal saline were administered intraperitoneally to groups of five mice. Each group was immediately placed in the cage for a ten-minute adjustment period which was followed by a fifteen-minute counting period. The concentrations of the solutions were adjusted so that the total volume administered was approximately 0.1 ml. An interval of two to three days was permitted between trials, and control tests utilizing normal saline solution or 30% propylene glycol solution were conducted periodically prior to initiating the drug studies and two to three days after the mice received the even-numbered dose of a compound. OM-518 and OM-520 (dissolved in 30% propylene glycol) were administered in doses varying between 10 and 100 mg./Kg., chlorpromazine was administered in doses between 1.25 and 10.0 mg./Kg., and sodium pentobarbital in doses varying from 5 to 30 mg./Kg.

Effects on Forced Motor Activity.—The effects of the compounds on forced motor activity of male, albino Swiss-Webster mice were studied using the Rotarod described by Dunham and Miya (2) and Kinnard and Carr (1). The apparatus consisted of a wooden rod, 1 1/8 inches in diameter and 36 inches long attached to a Gorrell and Gorrell electric kymograph (type S monodrum) placed horizontally. The entire unit was set on a suitable support and divided into six compartments utilizing cardboard disks, 7 inches in diameter. The mice were placed on the revolving rod (29 r. p. m.) in groups of six and the time that each animal remained on the rod recorded, with the maximum time set at one hundred

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OM-518 and OM-520 were kindly supplied by Dr. H. L. Daiell, Senior Vice-President, Lakeside Laboratories Inc., Milwaukee, Wis.
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TABLE I.—THE EFFECT OF CERTAIN CENTRAL NERVOUS SYSTEM DEPRESSANTS UPON MOTOR ACTIVITY OF MICE AS MEASURED BY THE PHOTOCELL ACTIVITY CAGE AND ROTAROD METHODS

		Photocell Activity Cage			Rotarod		
			Mean Activity Count/15 min. Period		Group Performance Time, sec. ± S. E.		PN Value for Trial 20[d]
Compound	Dose mg./Kg.	n[a]	± S. E.	PN[b] Value	n[c]	Trial 10[d]	Trial 20[d]	
Control	..	84	277 ± 12	100	48	106 ± 6	129 ± 6	100
OM-518	10	12	328 ± 13	118	6	138 ± 10	154 ± 10	119
	20	12	315 ± 24	113	6	128 ± 3	141 ± 11	109
	30	12	152 ± 10	54	6	154 ± 10	166 ± 11	128
	40	12	174 ± 38	62	6	62 ± 8	106 ± 18	82
	50	12	162 ± 22	58	6	75 ± 18	106 ± 13	82
	60	..	.....	..	6	40 ± 13	70 ± 13	54
	75	12	80 ± 26	9	6	52 ± 12	72 ± 12	55
	90	..	.....	..	6	42 ± 10	63 ± 8	48
	100	6	0	0	6	21 ± 10	22 ± 10	17
Control	..	84	282 ± 10	100	48	126 ± 4	123 ± 8	100
OM-520	10	12	238 ± 19	84	6	106 ± 10	131 ± 10	106
	20	12	194 ± 6	68	6	132 ± 11	132 ± 11	105
	30	12	143 ± 22	50	10	118 ± 6	128 ± 6	104
	40	12	92 ± 20	32	6	55 ± 11	102 ± 7	82
	50	12	150 ± 29	53	6	66 ± 9	88 ± 7	75
	60	12	.....	..	6	51 ± 11	66 ± 16	54
	75	12	17 ± 3	6	6	59 ± 13	61 ± 13	49
	90	..	.....	..	6	53 ± 17	62 ± 19	50
	100	6	0	0	6	0	0	0
Control	..	60	240 ± 11	100	54	89 ± 5	119 ± 5	100
Pentobarbital sodium	5	12	255 ± 27	106	6	123 ± 13	148 ± 16	124
	10	12	249 ± 28	103	12	122 ± 14	140 ± 12	117
	15	12	248 ± 33	103	6	88 ± 7	137 ± 12	115
	20	12	233 ± 38	97	6	8 ± 4	56 ± 13	47
	30	8	42 ± 18	17	6	0 ± 0	0	0
Control	..	60	237 ± 10	100	54	77 ± 6	107 ± 6	100
Chlorpromazine	1.25	12	219 ± 10	92	6	68 ± 9	108 ± 1	100
	2.50	12	139 ± 17	58	12	58 ± 10	59 ± 10	55
	5.00	12	87 ± 12	36	6	27 ± 5	34 ± 9	31
	7.50	12	35 ± 5	14	6	14 ± 2	17 ± 3	15
	10.00	12	24 ± 8	10	6	10 ± 3	19 ± 2	17

[a] n denotes the number of groups (five mice per group) tested. [b] PN = per cent normal. [c] n denotes the number of groups (six mice per group) tested. [d] Number indicates minutes after injection.

and eighty seconds. It took approximately five days to train the mice to walk the rotating rod. Solutions of the compounds, normal saline, or 30% propylene glycol were then administered intraperitoneally in a volume of approximately 0.1 ml. Performance trials were conducted ten and twenty minutes following injection and an interval of two to three days was permitted between trials. The dosage range of the compounds were identical to that used in the photocell activity cage studies.

RESULTS

Effects on Spontaneous Motor Activity.—The effects of the compounds on spontaneous motor activity in mice are summarized in Table I. OM-518 and OM-520 elicited a gradual decline in activity in doses over 20 mg./Kg. Although 100 mg./Kg. produced complete arrest of spontaneous activity, no loss of righting reflex was observed. Chlorpromazine also elicited a gradual decline in spontaneous activity in the dosage range studied. No stimulation or ataxia was noted and the mice did respond to external stimuli at doses which practically abolished spontaneous activity. Low doses of sodium pentobarbital (5–15 mg./Kg.) produced responses slightly above control values, however the differences between the mean responses were not significant at the 0.05 level. Higher doses produced decreases in activity and the mice receiving 30 mg./Kg. of sodium pentobarbital did not respond to external stimuli.

Effects on Forced Motor Activity.—The effects of the compounds on Rotarod performance time are summarized in Table I. OM-518 and OM-520 did not markedly alter forced motor activity in doses under 30 mg./Kg. The administration of doses over 30 mg./Kg. produced a gradual decline in the ability of the animals to walk the revolving rod. Chlorpromazine produced a gradual decline in performance time as the dosage was increased. Five and 10 mg./Kg. of sodium pentobarbital produced an apparent increase in forced motor activity, however doses over 20 mg./Kg. markedly depressed the ability of the mice to perform in a coordinated manner and 30 mg./Kg. abolished all activity.

DISCUSSION

When the dose-response effects of the compounds on spontaneous and forced motor activity were plotted there was a marked difference in the pattern of the responses of pentobarbital, chlorpromazine, and the oxazolidone compounds. OM-518 (Fig. 2) and OM-520 (Fig. 3) produced marked depression of spontaneous activity in doses which did not alter forced motor activity. Doses of 30 mg./Kg. of OM-518 and OM-520 depressed spontaneous motor activity approximately 50% whereas this dosage of either compound did not depress forced motor

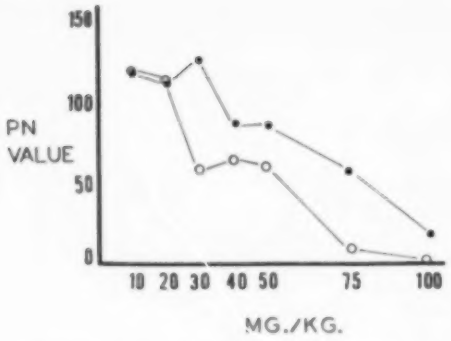

Fig. 2.—Effects of OM-518 on motor activity of albino mice. ○——○ Activity cage, ●——● rotarod. PN = per cent normal.

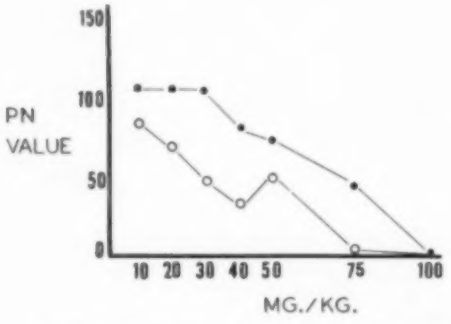

Fig. 3.—Effects of OM-520 on motor activity of albino mice. ○——○ Activity cage, ●——● rotarod. PN = per cent normal.

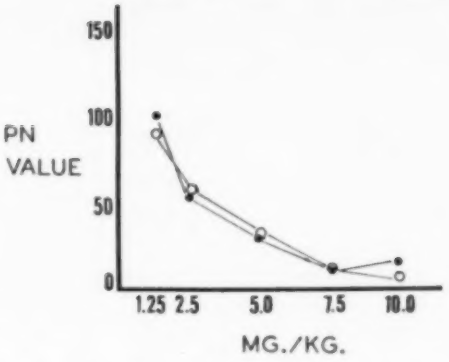

Fig. 4.—Effects of Chlorpromazine on motor activity of albino mice. ○——○ Activity cage, ●——● rotarod. PN = per cent normal.

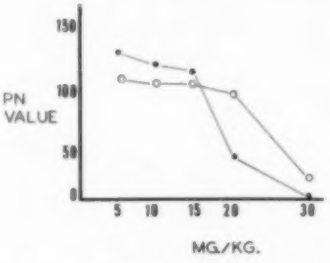

Fig. 5.—Effects of pentobarbital sodium on motor activity of albino mice. ○——○ Activity cage, ●——● rotarod. PN = per cent normal.

activity. Chlorpromazine produced approximately the same degree of depression of spontaneous activity and forced motor activity (Fig. 4). A 2.5 mg./Kg. dose of chlorpromazine produced a reduction in spontaneous and forced motor activity of slightly less than 50%. Sodium pentobarbital was mainly effective in depressing forced motor activity in that 20 mg./Kg. produced minimal effects on spontaneous activity while markedly depressing forced motor activity (Fig. 5). Although the two methods utilized in comparing these compounds are quite simple, it is very possible that these techniques can be utilized to differentiate sites of action. It would appear that those compounds acting mainly on the cerebral cortex would inhibit motor activity and coordination to a greater degree than spontaneous activity; whereas those compounds which induce an apparent depression of spontaneous activity through sites other than the cortex would produce minimal effects on motor activity. Carroll, *et al.* (4), have reported that 5-(3,5-dimethylphenoxymethyl)-2-oxazolidinone was a fairly potent muscle relaxant. Our experiments with the muscle relaxant α-isopropylbenzyl carbamate have shown that the compound produced marked depression of Rotarod performance. Kinnard and Carr (1) have also reported that meprobamate markedly decreased the ability of mice to perform in a coordinated manner. From the data obtained in this study it does appear that the two oxazolidone compounds produce a sedative type action with no visible relaxation of skeletal muscle, with the strong possibility that the site of action is subcortical.

SUMMARY

1. OM-518 and OM-520 produced marked depression of spontaneous activity in doses which did not alter Rotarod activity.

2. Chlorpromazine produced approximately the same degree of depression of spontaneous and forced motor activity.

3. Pentobarbital was more effective in depressing Rotarod activity than spontaneous activity.

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Hypertensive Properties of 4-Methyl-2-amino-pyridine

By JOSEPH P. BUCKLEY, WILLIAM J. KINNARD, MARIO D. G. ACETO, and ROBERT K. BICKERTON†

The cardiovascular effects of 4-methyl-2-amino-pyridine (RA-1226) were investigated in 18 anesthetized dogs. The mean rise in blood pressure produced by 1.0 mg./Kg. was 120 per cent and the blood pressure persisted at hypertensive levels for approximately one hour. Administration of 5 mg./Kg. of RA-1226, i. v., to spinal dogs produced a mean increase in blood pressure of 203 per cent and the pressures remained elevated for approximately one hour. Data obtained from dog cross-circulation experiments indicated that RA-1226 is a potent peripheral pressor agent possessing a fairly long duration of action and apparently void of central pressor activity.

VON HAXTHAUSEN (1) has reported that 4-methyl-2-amino-pyridine (RA-1226) produced a marked rise in the mean arterial blood pressure of cats and dogs and suggested that this action was due to peripheral vascular constriction. He also reported that the compound produced central analeptic effects, antispasmodic effects on isolated intestinal strips, and a contraction of the nictitating membrane of the cat. Gottstein, *et al.* (2), described in some detail the cardiovascular effects of RA-1226 in anesthetized dogs. They concluded that the compound produced a prolonged pressor effect due to peripheral vasoconstriction and possibly increased cardiac output. The compound also produced a concomitant decrease in cardiac rate. Since it was apparent that this compound is potentially useful in the treatment of severe circulatory collapse, we were especially interested in investigating the pressor activity of RA-1226 in anesthetized dogs having relatively low blood pressures. This present report deals principally with the hypertensive activity of 4-methyl-2-amino-pyridine in anesthetized hypotensive dogs.

EXPERIMENTAL

Cardiovascular Effects of RA-1226 in Anesthetized Dogs.—Mongrel dogs of both sexes were anesthetized with pentobarbital sodium, 35 mg./Kg., i. v. The left common carotid artery and both femoral arteries were isolated and 1,000 u./Kg. of heparin administered intravenously. The left common carotid artery was cannulated and connected to the inflow tubing of a Shipley-Wilson rotameter. The distal sections of both femoral arteries were cannulated and connected to the outflow from the rotameter. Flow rate was recorded on a Sanborn visocardiette and was obtained from either one or both femoral arteries depending upon the rate of flow. The proximal end of one of the femoral arteries was cannulated to record mean arterial blood pressure. Only those animals having relatively low blood pressures were selected for this study. Fresh aqueous solutions of RA-1226 were administered into the inflow to the femoral arteries.

RA-1226 on Experimentally Induced Hypotension.—Mongrel dogs were anesthetized by an intravenous dose of pentobarbital sodium, 35 mg./Kg. Arterial blood pressure was recorded from a carotid artery via a mercury manometer or a Statham pressure transducer. The trachea was cannulated to permit connection to a pulmotor. The animal was then placed dorsal side up and the spinal column exposed between C-2 and C-3 utilizing electrocautery. A laminectomy was performed and the dog placed on artificial respiration after sectioning of the spinal cord. The blood pressure was permitted to stabilize at a low level prior to the intravenous administration of RA-1226.

Peripheral and Central Effects of RA-1226.—The peripheral and central effects of RA-1226 on blood pressure were investigated in the dog cross-circulation preparation. The procedure used was a modification of the technique described by Taylor and Page (3) in that the spinal blood vessels (venous sinuses and vertebral arteries) were occluded between C-2 and C-3 utilizing a 21-gauge stainless steel wire. Two milliliters of tripelennamine (25 mg./ml.) was administered subcutaneously to the donor dog, twenty minutes before cross-circulation was established between the left common carotid artery of the donor and the two common carotid arteries of the recipient, and the two jugular veins of the recipient and the left jugular vein of the donor. Heparin sodium 2 to 4 ml. (1,000 u./ml.), was immediately administered intravenously to the donor animal. After the blood pressures of the donor and recipient had stabilized, 0.25 ml. of Lugol's solution, U. S. P. was injected into the arterial tubing to the recipient's head to saturate the thyroid glands with iodine. Ten microcuries of I^{131} (radio-iodinated serum albumin, human, Squibb) was administered into the arterial inflow to the recipient's head. Circulatory leakage between the body and the head of the recipient was determined periodically throughout the experiment by obtain-

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† George A. Kelly, Sr., Fellow.

Table I.—Effects of RA-1226 on the Blood Pressure of Anesthetized Dogs[a]

Dog No.	Dose, mg./Kg.	Original Blood Pressure, mm. Hg	Rise in Blood Pressure, mm. Hg	Change, %	Duration, min.	Heart Rate/min. Normal	Heart Rate/min. After RA-1226
1	1.0	48	58	121	90+	150	168
2	1.0	100	24	24	90+	...	...
2	2.0	112	24	21	48+	...	...
3	1.5	120	22	18	87+	160	155
4	1.0	30	68	227	10	150	170
5	1.0	...	...	...	...	130	140
6	1.0	82	62	77	90+	135	155
7	1.0	64	24	37	55	175	180
8	1.0	60	20	33	...	160	155
9	1.0	36	64	178	44+	168	200
10	1.0	18	78	433	65+	...	...
11	1.0	42	38	90	85+	...	...
12	1.5	70	36	51	15	135	140–165
13	1.5	102	32	31	36	...	...
14	1.5	46	44	96	15	155	185
15	1.5	108	58	54	18+	110	85
16	1.0	116	44	38	45+	...	...
17	1.0	88	26	30	70	175	190
18	1.0	46	74	161	67	140	175

[a] Anesthetic, pentobarbital sodium, 35 mg./Kg., i.v.

Table II.—Effects of RA-1226 on the Femoral Blood Flow in Anesthetized Dogs[a]

Dog No.	Dose, mg./Kg.	Original Flow, ml./min.	Flow after RA-1226, ml./min.	Duration, min.
1	1.0	4	15.3	5.2
2	1.0	13.5	68.2	2.0
4	1.0	4.2	19.1	2.0
4	1.0	8.5	23.5	4.5
5	1.0	16.0	21.2	4.5
6	1.0	46.7	87.0	18.0
7	1.0	21.2	32.6	1.5
8	1.0	19.1	50.0	3.0
9	1.0	10.0	32.6	1.5
14	1.5	5.5	9.0	1.0
15	1.5	13.5	64.5	0.5
16	1.0	17.1	38.2	0.5
17	1.0	13.5	30.4	1.0
18	1.0	13.5	35.5	1.0

[a] Anesthetic, pentobarbital sodium, 35 mg./Kg., i.v.

ing 3-ml. blood samples, from the left femoral vein of both animals, which were placed in a scintillation counter to determine minute radioactivity. Samples withdrawn prior to I^{131} administration served as controls. The activity of the control samples were subtracted from the readings of the subsequent samples and the per cent radioactivity in the recipient's peripheral circulation calculated as follows:

$$\frac{\text{corrected counts/min. in } R \times 100}{\text{corrected counts/min. in } R + \text{corrected counts/min. in } D}$$

In two experiments the donor dog was replaced by a Sigma motor pump (model TM-10) and a Pulmopak oxygenator (Abbott Laboratories), which provided oxygenated blood to the isolated head at the rate of approximately 80 ml./min. Fresh aqueous solutions of RA-1226 were administered into the arterial inflow to the recipient's head or into the recipient's right femoral vein.

RESULTS

Cardiovascular Effects in Anesthetized Dogs.—The effects of RA-1226 on arterial blood pressure and femoral blood flow are summarized in Tables I and II. Doses of 1.0 to 2.0 mg./Kg. produced marked pressor responses of relatively long duration in all experiments. The mean per cent rise in blood pressure produced by 1.0 mg./Kg. of RA-1226 was 120% with a duration of action of over one hour. The greatest per cent increase as well as the greatest actual increase in arterial pressure, expressed in mm. Hg rise in blood pressure, was observed in those preparations which had very low blood pressures prior to the administration of the pressor agent. Although RA-1226 did not markedly alter cardiac rate, it generally produced a slight increase in heart rate. RA-1226 produced an initial transient increase in blood flow which usually persisted from one-half to five minutes and was followed by a prolonged diminution in the rate of blood flow.

RA-1226 on Experimentally Induced Hypotension.—The effects of RA-1226 on the blood pressure of spinal dogs are summarized in Table III. RA-1226, 5 mg./Kg., i.v., produced an average peak increase in blood pressure of 203% (120 mm. Hg rise in pressure) which persisted approximately sixty minutes. Lower doses also produced marked pressor effects, however the duration of action was usually much shorter.

Peripheral and Central Effects of RA-1226.—The effects of RA-1226 on the blood pressures of the donor and recipient dogs in the cross-circulation studies are summarized in Table IV. Doses of 3 to 5 mg./Kg., via the femoral vein of the recipient, produced marked sustained pressor effects in the recipient dogs only; 5 mg./Kg., administered via the arterial inflow to the recipient's head, produced pressor effects in the donor dogs only. The data indicate that the pressor responses were entirely due to the peripheral actions of the compound and although RA-1226 has been reported to produce cen-

TABLE III.—EFFECT OF RA-1226 ON EXPERIMENTALLY INDUCED HYPOTENSION IN DOGS

Dog No.	Wt., Kg.	Sex	Initial Blood Pressure, mm. Hg	Hypotensive Level, mm. Hg	Dose, mg./Kg. i. v.	Peak Pressor Effect, mm. Hg	Increase, %	Duration, min.
1	5.9	M	125	68	5.0	175	157	45
				62	5.0	175	182	76
2	4.0	M	108	56	5.0	200	257	73
				70	5.0	160	128	..
3	6.2	F	100	76	5.0	220	189	81
				65	2.5	162	149	..
4	5.8	F	100	86	2.5	180	109	47
				82	2.0	148	80	..
5[a]	4.95	M	40	40	5.0	164	310	20
6	7.2	M	125/75	60/35	2.5	110/75	183/214	30

[a] Spinal cord intact.

TABLE IV.—EFFECT OF RA-1226 ON HYPOTENSION DUE TO SURGICAL MANIPULATION IN CROSS-CIRCULATION EXPERIMENTS

Expt. No.	Dog	Blood Pressure, mm. Hg	Dose mg./Kg.	Route	Max. Changes in Blood Pressure, mm. Hg	% Increase	Duration, min.
1	D[a]	80	3	i. v.-R	0	0	...
	R[a]	20			+10	50	20+
	D	80	3	i. v.-R	0	0	...
	R	30			+30	100	28+
2	D	60	4	i. v.-R	0	0	...
	R	30			+60	100	20+
3	D	60	4	i. a.-R	+45	75	20
	R	45			0	0	...
4[b]	R	70/30	5.0	i. a.-R	0	0	...
	R	75/35	5.0	i. v.-R	+47/+20	63/57	30+
5[b]	R	72/60	5.0	i. v.-R	+88/+70	122/140	50+

[a] D, donor dog; R, recipient dog. [b] Pump-oxygenator system utilized instead of donor dog.

tral analeptic effects, the blood pressure was not affected one way or the other by the action of the compound on central sites.

DISCUSSION

4-Methyl-2-amino-pyridine has been found to be a potent pressor agent possessing a relatively long duration of action. Although the compound produced pressor effects in anesthetized hypotensive dogs lasting over one hour, femoral blood flow was increased only for several minutes, which was followed by a decrease in the rate of flow indicating a marked increase in peripheral resistance. Since the compound does not appear to produce central hypertensive effects, the two probable mechanisms of action are direct stimulatory effects on the vascular system and/or the heart. The data suggest that the main site of action is the peripheral vascular system, with a possible secondary, minor effect on the heart. RA-1226 did not produce a decrease in cardiac rate as reported by Gottstein, *et al.* (2), and actually produced a slight increase in heart rate in the majority of dogs. This difference in the effects on cardiac rate could be due to the fact that most of the dogs utilized in the current report had extremely low blood pressures.

SUMMARY

1. The hypertensive activity of 4-methyl-2-amino-pyridine (RA-1226) was investigated in anesthetized hypotensive dogs, spinal dogs, and dog cross-circulation preparations.

2. RA-1226 produced marked pressor effects of extremely long duration in both anesthetized hypotensive dogs and spinal dogs.

3. RA-1226 produced a transient increase in femoral blood flow of relatively short duration followed by a prolonged, marked increase in peripheral resistance.

4. RA-1226 did not demonstrate any central hypertensive tendencies.

5. The hypertensive activity of RA-1226 was due entirely to the peripheral actions of the compound.

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Study of Various Methods for Detecting Leaks in Hermetically Sealed Ampuls

By W. J. ARTZ, W. T. GLOOR, Jr., and D. R. REESE

Six methods for detecting leaks in hermetically sealed ampuls were studied. The results indicated that the autoclave-dye method was the most selective of those studied. An outstanding advantage of this method is that heat-sterilizable ampuls are sterilized and tested for leaks simultaneously, thus eliminating one step in processing. It was found that dye immersion has no effect on the autoclave time required for sterilization of a batch of one thousand 1-cc. ampuls. Other leak-detecting methods studied were: vacuum-dye, pressure-dye, fluorescent penetrant solutions, external air pressure, and dye immersion after autoclaving. The attributes and limitations of each of these are discussed. Also included is a discussion, with photographs, of physical characteristics of ampul leakers.

THE PREPARATION of parenteral solutions is a great responsibility. From the first step to the last, the operator must continually remember that deviation from the prescribed technique may produce disastrous results in the patient. The primary purpose of ampuls is to provide individual sterile doses of solutions in separate containers that are sealed to prevent contamination by bacteria and other impurities. If there is a break in the seal of the ampul, contamination of the contents is almost inevitable; and very serious consequences may result if the solution is injected. This places a great responsibility on the test which is used to detect leaks in ampul containers.

A number of methods for detecting leaks in ampuls have been proposed in the parenteral industry. These run from the most frequently used method, vacuum-dye, to the helium-high frequency spark method (1). The effectiveness of the vacuum-dye and other tests used for commerical products has been questioned for quite some time. During stability studies we have seen more than one ampul that had passed the vacuum-dye test develop crystals at the tip as a result of small capillaries that had not been previously detected. Also, the literature cites several examples that point the finger of doubt at the methods currently used to detect leaks in ampuls. For example, visible cracks in ampuls of saline solution stored in a 2.5 per cent solution of phenol that is colored red with eosin (1:600,000) allowed the diffusion of appreciable amounts of phenol into the ampuls although sufficient dye did not pass in to be detectable (2). Macintosh (3) recorded the passage of alcohol into ampuls through cracks of molecular dimensions in the glass, no flaws being noticeable by visual inspection. Similarly, Steinberg (4) made a similar observation using thimerosal[1] tincture as the storage solution.

The present investigation was undertaken to study the vacuum-dye and other leaker-detection tests to determine which, of all the methods tested, is the most effective.

Before studying leaker-detection tests, it might be desirable to examine more closely the physical characteristics of ampul leakers. Figures 1–4 show the four major types of leaks in hermetically sealed ampuls: (*a*) The capillary, the most difficult to detect, consists of an opening in the glass extending from the tip of the seal to the base of the seal. It is usually narrower at the top. (*b*) A crack at the tip of an ampul is usually found at the base of the seal. This could be caused by sealing too much glass, jarring the ampul when it is still hot, or by general mishandling. (*c*) Cracks at the base of the ampul are probably due to mishandling by the glass manufacturer or by laboratory personnel, but the cause is unknown. (*d*) An incomplete seal is characterized by an opening, usually larger than a capillary, at the tip of an ampul. This is caused by having too low a flame temperature or by removing the ampul from the flame prematurely.

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[1] Merthiolate, Eli Lilly and Co.

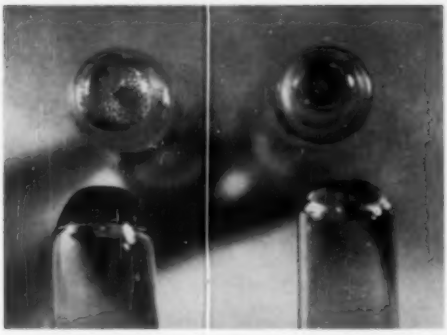

Fig. 1.—Photograph showing top and side views of capillaries found in hermetically-sealed ampuls.

Fig. 3.—Ampuls with cracked bases.

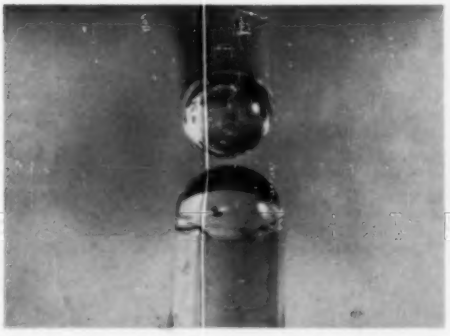

Fig. 2.—Tip-sealed ampuls showing cracks at the base of the seals.

Fig. 4.—Incomplete seals (top and side views).

EXPERIMENTAL

Fluorescent Penetrant Inspection.—The effectiveness of fluorescent penetrant inspection is based upon one of the most powerful forces in nature, capillary action. When ampuls are immersed in the penetrant solution (Zyglo-Pentrex[2]), the solution is pulled by capillary action into any defects that are present. A vacuum drawn on the ampuls will increase the penetrating action of the penetrant. The ampuls are then immersed in an emulsifier (Zyglo-Pentrex emulsifier[2]) which combines with the penetrant on the surface, but not in the defects, to make the penetrant water-washable. The mixture of surface penetrant and emulsifier is rinsed by a gentle spray of water. The spray should have the proper degree of force and velocity to remove only the penetrant on the surface and not that which is in the defect. After all of the surface penetrant is removed, the ampuls are immersed in an aqueous solution of a developer (Zyglo ZP-5[2]) which acts like a blotter to draw the penetrant in the defects to the surface for inspection. Before the ampuls are inspected, they are heated in a circulating hot air oven to warm the penetrant in the defects and thus enable the developer to function more effectively. Inspection, the step for which all others are preparatory, is carried out under high intensity black light in a darkened area. Any defects, capillaries, cracks, etc., will glow with a yellow-green brilliance.

Fluorescent-penetrant inspection is used in the electronic and related fields (5, 6) very successfully, but before it can be adopted for detecting leaks in hermetically sealed ampuls, several factors must be considered.

First, minor defects such as dimples, crevices, and other superficial flaws show forth the same fluorescence as a true leaker; therefore, a specially trained operator is necessary to distinguish between the two. Secondly, the penetration, emulsification, and rinsing procedures must be controlled rigidly or the desired results will not be obtained. For instance, if the penetrant-immersion time is too short, the penetrant will not enter all defects, and therefore will not detect all leakers. If the emulsification time is too short, all of the surface penetrant will not be removed, and the ampul will appear to be one mass of green fluorescence under inspection. If the emulsification time is too long, the penetrant in the defects will be emulsified and washed away during the rinsing procedure. If the rinsing spray is too forceful, it will remove the penetrant from the defects; if it is too weak, some of the surface penetrant will remain and interfere with inspection.

[2] Magnaflux Corp., Chicago, Ill.

Because some ampul rejects have cracks on the base or body of the ampul, it is imperative that the entire surface of the ampul be inspected if this method is used. To do so, each ampul must be inspected individually, which is a definite disadvantage in commercial production. Also, toxicity data on the penetrants, emulsifiers, and developers must be known, because it is conceivable that an ampul containing a leak could go undetected and yet contain sufficient amounts of testing materials to be dangerous if injected.

External Air Pressure.—The apparatus (Fig. 5) consists of a rubber stopper in which three holes have been bored, one on the side for air to enter and two on the top for ampuls to be placed, one tip down and one base down. The air which passes through the stopper will force its way into any cracks or openings that are present. Bubbles in the solution indicate that there is a leak present, and the ampul is rejected. The ampul must be rotated while it is being tested, so that the entire surface of the seal is in the direct path of the air, otherwise tiny cracks under the seal will not be detected. Also, it may be necessary to shake the ampul to force the liquid against the tip of the ampul. As mentioned earlier, it has been learned that some leakers contain cracks on the base of the ampul; therefore, it is necessary to test each ampul twice; once at the tip and once at the base.

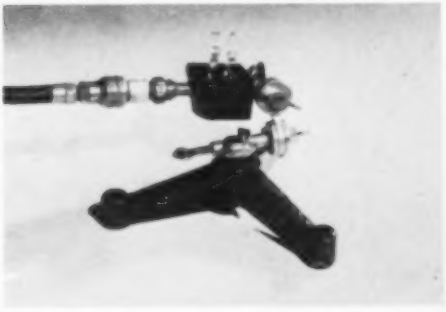

Fig. 5.—Apparatus used to detect leaks in ampuls by the external air pressure method.

This detection method was very effective in detecting tiny capillaries in ampuls that were sealed with a minimum amount of glass. Evidently the pressure that was used, up to 50 lb. per square inch, was forceful enough to break through the glass walls of improperly sealed ampuls. Approximately 600 leakers from a batch of 4,000 ampuls passed an unstandardized vacuum-dye test but were detected by this method.

A disadvantage of this method, in addition to the inconvenience and impracticality of testing ampuls twice, is that it does not detect cracks that might occur on the body of the ampul. To test the entire surface of ampuls, a method must be used in which the ampuls are immersed in a test solution.

Vacuum-Dye Method.—This is probably the most widely used leaker-detection test in the parenteral industry today. It consists of immersing ampuls in a dye-bath, applying a vacuum, and subsequently releasing the vacuum, forcing the dye solution into ampuls that are not completely sealed. The apparatus used in our tests (Fig. 6) is a standard laboratory desiccator which was connected to an aspirator that draws a vacuum of 25–27 in.

The efficiency of this test has been increased greatly by standardizing each step in the procedure. Before this study was begun, any dye that was available was dissolved in water until a concentrated color was obtained. This solution was then used in the leaker tests. Now, the following solution is used:

FD&C Red No. 1	0.50%
FD&C Red No. 2	0.10%
Duponal C[1]	0.25%
Purified water, *q. s. ad.*	100.00%

Only a fraction of a drop of this solution in a 1-cc. ampul containing a colorless aqueous solution will discolor the contents so that it is easily detected. The Duponal C lowers the surface tension at the crack or capillary and increases the penetrating properties of the solution. Alcohol in place of water did not increase the effectiveness of this solution. It was found that red was more easily detected than blue, green, yellow, or orange by the personnel in the ampul department; however, one of ten persons tested could not detect red or pink in normally colorless ampuls. It was later learned that this person was color blind to red and green. It is now a standard practice to test every person for color blindness before he is permitted to test for leakers.

Fig. 6.—Laboratory desiccator used in the vacuum-dye method for detecting leaks in ampuls.

Before this study, the vacuum was released every three minutes for three times. This study, however, shows that the most effective procedure is to release the vacuum every ten minutes for three times. It also shows that releasing the vacuum sharply is more effective than releasing it slowly, and that best results are obtained when the vacuum chamber is completely filled with dye solution.

It has been reported that pretreatment with heat or with an ultrasonic generator aids in opening up the leaks so that the effectiveness of the vacuum-dye test is increased (7).

Standardization of the vacuum-dye test has greatly increased its effectiveness; however, it has been found that this method is not as effective as the

[1] E. I. Du Pont de Nemours and Co.

autoclave-dye test which is discussed and compared with the vacuum-dye test later in this report.

Pressure-Dye Method.—This is essentially the same as the vacuum-dye test except that a pressure, instead of a vacuum, is exerted on the ampuls in a chamber filled with dye solution. The apparatus consists of a steel tank attached to a hand pump, on which can be obtained pressure up to 200 lb./sq. in. The standard red dye solution, which was described earlier, was used as the detecting media.

In comparison tests it was learned that the vacuum method was more effective than the pressure method. In each of eight tests at least one leaker was detected by the vacuum method that was not detected by the pressure method. Also, in five additional tests not one leaker was found by the pressure test after the ampuls were inspected by the vacuum test. The conditions used in the pressure tests ranged from 50 lb. pressure for fifteen minutes to as high as 180 lb. pressure for ten minutes. It was observed that pressure even as high as 150 lb./sq. in. did not improve the effectiveness of the test.

Autoclave-Dye Method.—In the autoclave-dye method, the ampuls are autoclaved while they are immersed in dye solution. They are then rinsed and inspected in the usual manner. It was found that it makes little difference whether the ampuls are inspected immediately or whether they are allowed to cool to room temperature in the dye solution. A cyclomatic autoclave made by the American Sterilizer Co. was operated at slow exhaust during this study. If the autoclave is exhausted too rapidly, the rate of loss of pressure will exceed the rate of reduction in temperature of the dye solution, thereby causing the solution to boil and possibly overflow. It is important that the ampuls be completely immersed in the dye solution throughout the autoclave cycle; otherwise a leaker may escape detection. This can be accomplished by tying the ampuls in sacks of cheesecloth.

The autoclave-dye and the vacuum-dye methods were compared by subjecting ampuls first to one test then to the other to determine which is the more selective. It was found, in ten separate tests, that the vacuum-dye method detected no leakers in ampuls that were previously tested by the autoclave-dye method. On the other hand, it was found that the autoclave-dye method detected leakers in five out of 10 batches that were previously tested by the vacuum-dye method. It is evident that the autoclave-dye method is more selective than the vacuum-dye method; but the difference in selectivity is not so great as shown here. We have found that subsequent autoclaving of ampuls will detect additional leakers; therefore, the second autoclaving in the autoclave-dye method accounts for some of the additional leakers. It is not at all likely that it would account for all of the additional leakers. We feel that autoclaving does not necessarily detect previously undetected leakers but that it, by nature of its extreme conditions, forces leaks in ampuls that may be weak, strained, or poorly sealed.

In addition to being more selective than the vacuum-dye method, the autoclave-dye method is more practical because it combines the detection of leakers with the sterilization of ampuls, thereby eliminating one step in processing. To determine the effect of dye immersion on the time required for sterilization, a study was conducted in which 1-cc. ampuls were filled with trypticase soy broth which was freshly inoculated with *B. subtilis* spores in a concentration of 0.08 cc. per 800 cc. of media. One thousand ampuls tied in cheesecloth sacks were placed in a tray containing dye solution and autoclaved for either twenty, thirty, or forty-five minutes. One hundred ampuls were kept free of the dye solution during autoclaving to act as a control. Following autoclaving the ampuls were incubated at 30° for ten days. They were then inspected for turbidity which would indicate bacterial growth resulting from insufficient autoclave time. No contaminated ampuls were found in any of the groups, indicating that there is no difference between ampuls autoclaved in dye and without dye. We have found that by using the equipment described above twenty minutes is sufficient autoclave time to sterilize 1-cc. aqueous ampuls that are immersed in dye solution.

Before this method is used in commercial production it will undoubtedly require some modification to handle the larger volume of ampuls.

Dye-Immersion After Autoclaving.—When ampuls are immersed in a dye-bath immediately after autoclave sterilization, those containing leaks will pull in dye as they cool to room temperature. This is a very simple method and has been found to be at least as effective as the vacuum-dye method. In comparison tests, no leakers were detected by the vacuum-dye method in 12 batches of ampuls that were previously tested by dye-immersion after autoclaving. In 10 other tests this method did not detect additional leakers after the ampuls had been tested first by the vacuum-dye method.

If the ampuls are not immersed in the dye-bath immediately after autoclaving, some leakers will draw in air when they cool, and therefore will be unable to draw in dye when they are immersed.

CONCLUSIONS

The results of this study indicate that the most effective method for detecting leaks in heat-sterilizable ampuls is the autoclave-dye method. This method has the added advantage of the ampuls being sterilized at the same time that they are tested for leaks, thus eliminating one step in processing.

It also appears that a satisfactory leaker-detection method will result if ampuls are immersed in a dye-bath immediately after autoclaving.

For ampuls that cannot be sterilized by autoclaving, it is recommended that the vacuum-dye method, standardized as in this study, be used. Although not as effective as the autoclave-dye test, this method is moderately reliable.

Regardless of the leaker-detection method used, one cannot be sure that all of the leakers (100% of them) are detected. It is important then, that every possible means be taken to avoid leakers. Following are recommendations which may help reduce the number of leakers.

(*a*) The method of sealing should be used that will give the most satisfactory seal. It appears to be the consensus in the parenteral field that pull-sealing is more dependable than tip-sealing.

(*b*) The sealer should discard improperly sealed ampuls immediately. If they continue through processing, they may be overlooked.

(*c*) Ampuls should always be handled gently to avoid cracks caused by mishandling.

(*d*) A standard should be made for properly sealed ampuls, and any ampul that does not live up to this standard must be discarded.

SUMMARY

1. A brief discussion of the types of leaks found in hermetically sealed ampuls is presented, with photographs.

2. Six methods for detecting leaks in ampuls are discussed, including the attributes and limitations of each.

3. The fluorescent-penetrant inspection method has many factors which must be controlled before it can be adapted for ampul-leaker inspection. Several of these are listed.

4. External air pressure is quite effective in detecting leaks at the tip and base of ampuls but it fails to detect cracks which are on the body of the ampuls.

5. Because cracks can occur at any place on an ampul, a method in which the entire ampul is immersed in a detecting media appears generally to be the method of choice.

6. Standardization of the vacuum-dye method has greatly increased its effectiveness. The means of standardizing this method are presented.

7. The selection of the type of dye to use for leaker detection depends on the color-correction situation in the eyes of the inspectors. All inspectors should undergo a test for color blindness. The formula of the dye solution found to be very effective in this study is listed.

8. The vacuum-dye method was found to be more effective than the pressure-dye method.

9. The autoclave-dye method was found to be more effective than the vacuum-dye method.

10. Immersion in dye solution has no effect on the autoclave time required for sterilization of a batch of one thousand 1-cc. ampuls.

11. The autoclave-dye method eliminates one step in processing because it sterilizes the ampuls and tests them for leakers at the same time.

12. Dye-immersion after autoclaving is at least as effective as the vacuum-dye method and may be as effective as the autoclave-dye method.

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Production of Hypertensive Rats by Experimental Stress

By WILLIAM J. HUDAK† and JOSEPH P. BUCKLEY

Twenty-four Wistar rats were subjected to three types of stress, four hours a day, four days per week, for a period of thirty-five weeks in an attempt to produce hypertensive rats which could be utilized in the investigation of antihypertensive compounds. The blood pressures of the stress animals gradually increased reaching a maximum mean of 152 mm. Hg for males, at the end of the twenty-second week, and 151 mm. Hg for females, at the end of the twenty-eighth week. Fifteen rats (8 males and 7 females) died before the study was terminated with the greatest number of fatalities occurring between the twelfth and eighteenth week.

Weiss (1), Alexander (2), Saul (3), and Dunbar (4), have called attention to the unresolved psychologic conflicts which give rise to chronic emotional tension, and which they believe are specifically related to hypertension in man. As an experimental approach to this problem, Farris, *et al.* (5), and Medoff and Bongiovanni (6), subjected rats to audiogenic stimulation (air blasts five to ten minutes daily) and obtained systolic blood pressures of over 180 mm. Hg. Selye (7) suggested that the arterial lesions and hypertension that develop in rats following stress are the result of formidable physical stimulation leading to exhaustion. Shapiro and Melhado (8) subjected rats to varying types of stress, both individually and in groups, and after twelve weeks concluded that psychologic disturbance did not increase the level of blood pressure.

The object of this present study was to produce hypertensive rats for investigational purposes by means other than surgery or cortical steroid administration. It was felt that if hypertensive animals could be produced by subjecting them to variable stress conditions, the resulting pressor responses would be more physiological than the standard methods and that hypotensive responses produced by antihypertensive drugs in these animals would more closely correlate with expected responses in the hypertensive human.

METHODS

Twenty-four albino Wistar rats (12 of each sex) weighing approximately 150 Gm. were subjected to the stress conditions for four hours a day, four days a week, for a period of thirty-five weeks. The following forms of stress were utilized: (*a*) Flashing bright lights utilizing 150-w. indoor reflector spot lights. Four lights were placed around each cage, approximately 18–24 inches from the center of the cage. Each light flashed 30 times per minute for a one-second interval, with two lights flashing simultaneously. The intensity of the light was approximately 25 foot candles. (*b*) Audiogenic stimulation produced by tape recording noxious sounds (compressed air blasts, bells, buzzers, and tuning fork impulses) at five-minute intervals for one-half minute periods and then highly amplifying them upon reproduction. The intensity of the audiogenic stimulation at the site of the rat was approximately 100 decibels. (*c*) Oscillation at the rate of 60 times per minute.

The stresses were applied as follows: *a* plus *b* for a two-hour period, *a* plus *c* for a one-hour period, *b* plus *c* for a one-hour period. The stress conditions were altered daily to prevent acclimation to the stimuli. Following the daily stress period, the rats were maintained under standard conditions. Six control rats (three of each sex) were not subjected to any of the stresses. Systolic blood pressures were obtained every other week by means of the photoelectric tensometer.[1] Both stress and control males were placed on 0.9% sodium chloride drinking solution on the fifteenth week.

RESULTS AND DISCUSSION

The animals subjected to the stimuli became hyperactive and irritable and exhibited excessive salivation, lacrimation, urination, and defecation. Fifteen rats (eight males and seven females) died before the study was terminated, with the greatest number of fatalities occurring between the twelfth and eighteenth weeks. The animals were observed to be in a state of rigor almost immediately following death. No fatalities occurred in the control group.

The blood pressures of the experimental female rats gradually rose for six weeks (Fig. 1). Following a temporary period of apparent acclimation, the pressure continued to rise to a maximum mean pressure of 151 mm. of Hg at the end of the twenty-eighth week. When the data were subjected to the "*t*" test, the difference between the mean blood pressure of the control rats and mean pressure of the stress rats was significant at the 0.001 level. After reaching this peak, the blood pressure decreased in the next seven weeks to a mean of 120 mm. Hg, indicating an apparent acclimation to the stimuli. The blood pressures of the male rats followed a pattern similar to the females (Fig. 2). The maximum mean blood pressure of 152 mm. Hg was reached at the twenty-eighth week (the difference between the means was significant at 0.001 level) after which the pressure decreased to a mean of

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† Present address: Department of Pharmacology, William S. Merrell Co., Cincinnati, Ohio.

[1] Metro Industries.

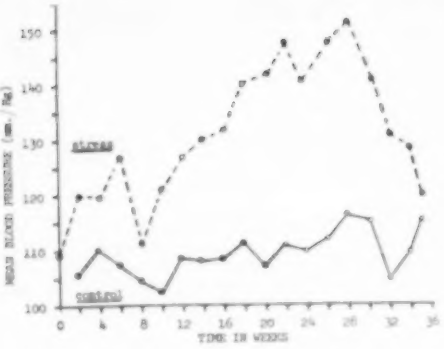

Fig. 1.—The effects of experimental stress on the blood pressure of female rats.

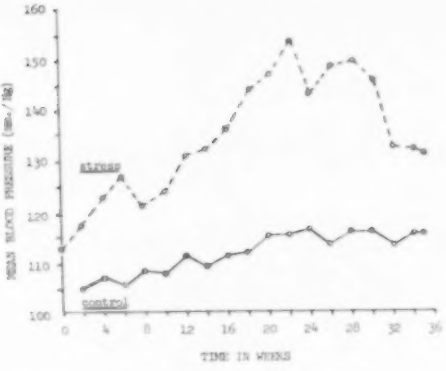

Fig. 2.—The effects of experimental stress on the blood pressure of male rats.

125 mm. of Hg. Sodium chloride drinking fluid failed to increase the pressor response, as the pressure of the control males remained unchanged and the degree of blood pressure increase between the males, which received saline, and the females, which received tap water, were approximately the same.

The animals surviving the experiment were sacrificed and their kidneys and adrenal glands removed for histological examination.[2] The kidneys of the stress animals contained pigmented granules which appeared in masses, mainly in the cortical layers; the tubules appeared greatly constricted, and the kidney cells were enlarged. The adrenal glands of the stress animals contained individual pigmented granules which appeared only in the zona fasciculata of the cortex.

Although this investigation was originally initiated as a pilot study, it was evident that the animals subjected to variable stress conditions could be utilized in investigating hypotensive compounds. The acclimation which occurred after a period of approximately twenty-five weeks might be prevented by substituting different stress conditions prior to that time. It was also evident that the rats had undergone marked behavioral changes and that these animals could be utilized in the investigation of psychotropic compounds.

[2] The authors are indebted to Dr. M. Jollie, Department of Biological Sciences, University of Pittsburgh, for interpreting the histological slides.

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Assay of Certain Alkaloids in Nonaqueous Solution

By S. M. WANG and R. W. HUNTER

A method is described for the assay of alkaloids or alkaloidal salts either in the pharmaceutical preparation or plant extract which is based on separating the alkaloids by ion exchange resin and determination of them in the nonaqueous solution, either by visual indicator method or potentiometry.

THIS PAPER deals with the application of ion exchange adsorption and nonaqueous titration for the determination of alkaloids (1, 2). A small quantity of alkaloids from 0.01 to 0.1 meq. can be extracted out from the aqueous solution by ion exchange resin such as Amberlite IRC-50.[1] The alkaloids can be easily eluted from the resin by treating them with nonaqueous solution, such as glacial acetic acid. The resulting solution can be directly titrated by standard perchloric acid solution, either with visual indicator or potentiometry.

Amberlite IRC-50 has been employed, because of (*a*) its weak cation exchange character, (*b*) its total capacity of exchange of 10 meq. per Gm. of the solid, and (*c*) the high efficiency with which this resin can easily be regenerated with acid.

EXPERIMENTAL

Materials and Reagents.—Glacial acetic acid U. S. P.; methyl violet indicator, 0.2% in chlorobenzene; perchloric acid, 0.01 *N*; Amberlite IRC-50(H), and Beckman model G pH meter.

Assay of Alkaloids.—Weigh accurately about 5 to 50 mg. of alkaloids or alkaloidal salts, either in the form of tablet or solution, and dissolve in 25 ml. of water. Add a drop of glacial acetic acid for free alkaloid. Filter off any inert materials and slowly add 10 ml. of mercuric acetate solution (10 mg. per ml.) to the mixture; a precipitate will form. Weigh a 1-Gm. portion of pretreated Amberlite IRC-50(H) add to the above solution, and shake the mixture on a shaking machine for thirty minutes. Filter off the solid, test the filtrate for complete extraction by using alkaloidal reagent. Remove the excessive amount of water and place the solid in 25 ml. of glacial acetic acid, warm at 60° for ten minutes, shake for another thirty minutes, then add two drops of methyl violet indicator. Titrate the solution until the disappearance of purple tinge or titrate the solution potentiometrically with a Beckman model G pH meter.

Assay of Alkaloids from Plant Extract.—Weigh accurately a portion of crude extract containing 5 to 50 mg. of alkaloids, place in a Soxhlet apparatus, add 2 ml. of ammonium hydroxide solution, and completely extract with an immiscible solvent such as chloroform. Evaporate the solvent to one-fifth of the original volume. Extract the alkaloids with three 10-ml. portions of 5% acetic acid and evaporate the mixture until free from chloroform. Follow the procedure as described in the above assay. Titrate the final solution potentiometrically with a Beckman model G pH meter. The assay results are given in Table I.

TABLE I.—ASSAY OF ALKALOIDS BY ION EXCHANGE SEPARATION AND NONAQUEOUS TITRATION

Alkaloids	Amount Added, mg.	Amount Found,[a] mg.
Atropine	50.00	50.30
	20.00	20.15
	5.00	4.98
	S. D. 0.1	
Brucine	50.00	50.25
Ephedrine hydrochloride	50.00	50.05
Homatropine hydrobromide	50.00	50.75
Pilocarpine hydrochloride	50.00	50.29
	20.00	20.35
	5.00	5.20
	S. D. 0.14	
Strychnine sulfate	50.00	50.90
Atropine sulfate tablet (Eli Lilly and Co.)	0.325 per tab.	0.320 per tab.
Ephedrine sulfate solution (Parke Davis and Co.)	0.05[b] per ml.	0.048[b] per ml.
Scopolamine hydrobromide tablet (Brewer and Co., Inc.)	0.4 per tab.	0.38 per tab.
Belladonna extract powdered U. S. P.	12.5	12.0

[a] Average of three determinations. [b] Quantity given in grams.

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[1] The Amberlite IRC-50(H) used in this study was generously supplied by Rohm & Haas Co., Philadelphia, Pa.

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Auto-oxidation of Apomorphine

By P. N. KAUL† and E. BROCHMANN-HANSSEN

The auto-oxidation product of apomorphine has been isolated. On the basis of elemental analysis, spectral evidence, as well as solubility and chromatographic behavior, it appears to be devoid of nitrogen, possibly an 8-substituted phenanthrene-3,4-dione. A scheme is suggested for the degradative process. The decomposition product has no emetic property.

IT IS WELL KNOWN that aqueous solutions of apomorphine rapidly undergo oxidative changes and turn green. This decomposition is oxygen- and pH-dependent (1–3), but nothing is known about its mechanism. Several antioxidants have been suggested to prevent discoloration of solutions (4, 5) and also to improve recovery of the alkaloid in the assay of solutions containing apomorphine (6). Gorrell and Gray (7) and Ponte (8) have reported that the development of green color in old solutions is not accompanied by any qualitative or quantitative changes in pharmacological activity.

The present study was undertaken in an attempt to clarify the reaction mechanism of the *in vitro* decomposition of apomorphine.

EXPERIMENTAL

A solution of apomorphine hydrochloride U. S. P. in water (about 0.5%) was allowed to stand for a few days in a separatory funnel. During this time, it turned dark bluish-green, and a dark-colored deposit was formed on the sides of the container. The solution was extracted several times with chloroform, and the combined chloroform extracts were washed repeatedly with 1 *N* hydrochloric acid to remove apomorphine and any other amine forming water-soluble salts. Finally, the solution was washed with water and dried over anhydrous sodium sulfate. The chloroform was removed under reduced pressure at a temperature not exceeding 40°, and the residue was crystallized several times from a mixture of chloroform and ethyl acetate (1:10). The dark blue, microspheroidal crystals softened at 165° and melted with decomposition at 195°.

Subsequently, the same product was obtained in shorter time by bubbling air through an aqueous solution of apomorphine hydrochloride, extracting the green color with chloroform, and then following the procedure described above.

Qualitative elemental analysis of the isolated blue substance gave a negative test for nitrogen.

Paper chromatography on buffered paper (pH 3–7) with water-saturated butanol as the mobile phase showed that the R_f value of the isolated decomposition product was independent of pH ($R_f = 0.85$).

The electronic absorption spectrum of an alcoholic solution was found to be pH-dependent, suggesting the presence of a dissociable acidic function. The changes in the ultraviolet spectrum accompanying changes in pH were small and reversible in the range of pH 1 to 9. An irreversible change occurred at pH above 9.

Figure 1 illustrates the infrared spectrum of the oxidation product (upper curve) as compared with that of apomorphine (lower curve).

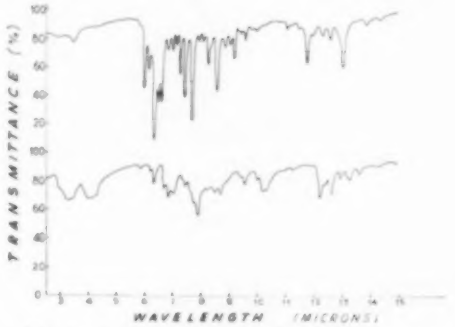

Fig. 1.—I. R. absorption spectra of apomorphine (lower curve) and its auto-oxidation product (upper curve).

In one trial, a dog weighing about 3 Kg. was given intravenously 5 mg. of the oxidation product of apomorphine. No emesis occurred, nor did the animal show any excitement or increased metabolic activity. In contrast, a similar dose of apomorphine invariably produces emesis and general stimulation in dogs of comparable weight.

DISCUSSION

The oxidation product is devoid of nitrogen as revealed by its elemental analysis and supported by its solubility characteristics, chromatographic behavior, and by the absence of any absorption band around 4.1 μ where the $\equiv N^+—H$ group usually absorbs.

The decrease in absorption around 3 μ and the presence of a strong band at 6 μ indicate that the catechol function may have been oxidized to *ortho*-quinone. In contrast, the apomorphine spectrum shows an absorption band at 4.05 μ, indicating the presence of a charged nitrogen. The broad peak at 3.25 μ and the shoulder at 2.9 μ are probably due to C—H stretch and hydrogen bonded hydroxyl groups.

The chain of degradative reactions most likely starts at the catecholic function of the apomorphine molecule. Since the rate of degradation increases with an increase in pH, dissociation of at least one of the two phenolic hydroxyl groups must be necessary for initiating the auto-oxidation. The preferred steric orientation of the apomorphine molecule favors hydrogen bonding of the hydroxyl group of the 4-position with oxygen at position 3 (I). The dissociation would, therefore, occur in the hydroxyl group in the 3-position. The resulting phenolate (II) would make the molecule susceptible to atmospheric oxygen and potentiate further electronic

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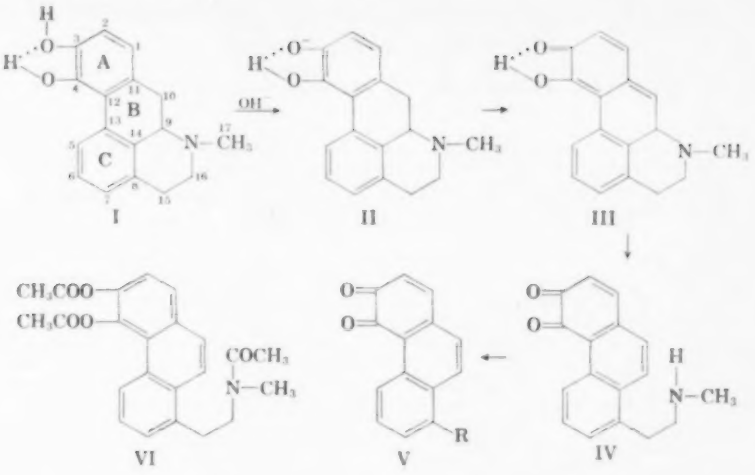
† Present address: University of Melbourne, Department of Pharmacology, Carlton N 3, Victoria, Australia.



rearrangements (11). On the other hand, diacetyl apomorphine, in which no such dissociation can take place, is stable in aqueous solutions (9).

One of the characteristic structural features of apomorphine is a dihydrophenanthrene system, which under appropriate driving forces would tend to oxidize to phenanthrene, thermodynamically a more stable structure because of its increased resonance. This would favor opening of the piperidine ring at a point between the tertiary nitrogen and ring B. With further oxidation, the catechol function would go to an *ortho*-quinone structure (IV). Such a compound would show a high degree of conjugation, with possible forms of resonance exhibiting a separation of charge. This would account for the intense color of the oxidation product.

Since the isolated oxidation product is free of nitrogen, this element must be eliminated, possibly as methylamine or amine oxide, during the continued oxidation process. This would result in phenanthrene-3,4-quinone substituted in position 8 (V). The substituent, although not yet established, might be a vinyl group.

It is well known that the bond between the tertiary nitrogen and ring B of apomorphine is weak and easily broken by treatment with various reagents. Thus, when apomorphine is treated with excess benzoyl chloride or acetic anhydride, fission of the nitrogen-containing ring occurs, the products being tribenzoyl apomorphine and triacetyl apomorphine, respectively (9, 10). Both of these compounds have been shown to be aromatic phenanthrene derivatives (VI). Treatment with cyanogen bromide likewise results in the opening of the nitrogen ring (11).

Hofmann's degradation of the dimethyl ether of apomorphine gives 3,4-dimethoxy-8-vinylphenanthrene which can be further oxidized to 3,4-dimethoxy-phenanthrene-8-carboxylic acid (10, 12). One might argue that the method of Hofmann's degradation uses rather rigorous treatment quite different from the situation that exists during *in vitro* auto-oxidation. However, when the two phenol groups of apomorphine are not protected by methylation or esterification, the same or similar reactions may require much less drastic conditions.

It is of interest to note that the colored oxidation product of apomorphine has no emetic property. This observation contradicts the findings of Gorrell and Gray (7). However, it supports the work of Tiffeneau and Porcher (9) who reported that triacetyl apomorphine (VI) possessed no emetic property, whereas diacetyl apomorphine, being converted in the body to apomorphine, was active.

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Physicochemical and Pharmacological Properties of Beef Muscle Hydrolysate

By B. CALESNICK and D. MILLIGAN

A hydrolysate of beef muscle, which is used therapeutically, was studied in order to establish physicochemical constants and the relative degree of toxicity. The presence of polypeptides was established and a fair degree of consistency of composition was found in the lots tested. The acute toxicity studies revealed the safety of this preparation when used in the suggested dosages.

THIS STUDY was undertaken to determine the physicochemical and pharmacological properties of a preparation which, according to the manufacturer, is produced by "the enzymatic hydrolysis of beef muscle extract" (1). It is further stated that "three distinct hydrolytic procedures are involved and the protein fractions removed by established routine methods" (1). This hydrolysate has been used clinically for many years and sold under the trade names of Pro-Zyme and A.N.F. (antineuritic factor). The present name is Viro-Zyme[1] and is marketed as a "5% solution of fractionated proteo-ribonucleotides complex in 0.5% phenol and 0.2% benzyl alcohol." It is administered intramuscularly and is recommended for various types of neuritic pain.

EXPERIMENTAL

Materials

Various commercial lots were used in this study, since it was considered important to compare these lots in order to determine consistency of composition and reproducibility of results.

Procedures

Physicochemical.—The pH of the solutions was determined in duplicate, with a Beckman model N pH meter.

Total nitrogen and nonprotein nitrogen were determined by the micro-Kjeldahl method (2) using trichloroacetic acid as the protein precipitant.

Absorption Spectra.—The ultraviolet and visual absorption curves were determined in triplicate using the Beckman model DU spectrophotometer. The blanks and diluent consisted of 0.5% phenol and 0.2% benzyl alcohol in distilled water. A concentration of 250 mcg./ml. was used in the former study and 500 mcg./ml. was used in the latter study. Absorption curves were also obtained using the fluorometric method. The Amineo-Bowman spectrophotofluorometer was used for the measurement of fluorescence and for recording of activation and fluorescence spectra. The concentration of material used in this study was 2.5 mcg./ml.

Paper Chromatography.—Resolution of chemical fragments of this preparation was attempted by paper chromatography. The ascending technique was used with the *n*-butanol-acetic acid system and Whatman No. 1 filter paper (3). The strips were developed with ninhydrin or examined directly under U. V. light. Absorbances were determined in the patterns developed with ninhydrin.

Paper Electrophoresis.—Using the method described by Jefferis and Everts-Suarez (4) patterns were obtained using Whatman No. 1 filter paper and barbital buffer, pH 8.6. These patterns were developed in the dark with ninhydrin.

Pharmacological.—In these studies, the acute LD_{50} was determined in white Swiss mice with an average weight of 20 Gm. Initial observations revealed that up to 1 ml. of the commercially available preparation failed to kill any of these animals when injected intraperitoneally. Hence, in order to increase the dosage within a feasible volume, a 25% concentration was supplied by the manufacturers. Controls were given an equivalent volume of normal saline.

In order to determine if Viro-Zyme (v-z) produced hemolysis *in vitro*, a study was set up using 10 test tubes containing 2 ml. of normal saline. To each tube was added 0.1 ml. of a 50% suspension of washed human erythrocytes and the following amounts of v-z:

	Tube No.	Ml.
5% v-z	1	0.1
	2	0.2
	3	0.3
	4	0.4
	5	0.5
25% v-z	6	0.1
	7	0.2
	8	0.3
	9	0.4
	10	0.5

All tubes were incubated for thirty minutes at 37° and then inspected grossly for evidence of hemolysis. Tubes containing the various dilutions of the hydrolysate were used as blanks.

Results

Physicochemical.—The pH of the various lots varied between 5.35 and 5.48.

The total nitrogen was found to be 9.27 mg./ml. with a nonprotein nitrogen of 8.13 mg./ml. The nitrogen, precipitated by trichloroacetic acid, was calculated as the difference between the above two values and was found to be 1.14 mg./ml. All of the various lots gave a positive biuret reaction.

The absorption spectra in the ultraviolet range (between 300 and 530 mμ) showed a slightly different slope between two lots. On the other hand, the absorption spectra of the two different lots coincided between 400 and 700 mμ. The spectrophotofluorometer recorded activation at 370 mμ and fluorescence at 450 mμ.

Representative paper chromatograms from each

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[1] Supplied by Quaker City Pharmacal Co., Philadelphia, Pa., and Marcen Laboratories, Inc., New Rochelle, N. Y.

lot differed slightly from each other. It was observed that as many as seven fractions were discerned under U. V. light. No attempt was made to determine if they were single substances or mixtures. The following R_f values were obtained: 0.14, 0.20, 0.27, 0.33, 0.38, 0.45, and 0.50. The relative concentrations of each fraction, demonstrated by densiometric studies, revealed the highest concentration at R_{f3} and the lowest at R_{f7}.

Paper electrophoretic patterns confirmed the similarity of the lots.

Pharmacological.—The LD_{50} in the white mice was found to be 8.2 ± 1.2 (S. E.) Gm./Kg.

In the *in vitro* test for hemolysis, no evidence of hemolysis was discerned in any of the tubes.

SUMMARY

In studying the chemical properties of this hydrolysate, it has been established that a number of ingredients are present. Since the chromatograms and electrophoretic patterns reacted with ninhydrin, there is presumptive evidence of a free α-amino acid such as is found in proteoses, peptones, and peptides. Further evidence was obtained by a positive biuret reaction which confirms the presence of polypeptide linkage.

It is generally assumed that the nonprotein nitrogenous compounds are separated from the protein by the use of a protein precipitant. However, it is possible that the fragment of nitrogen precipitated by the trichloroacetic acid in these studies represent some of the larger polypeptide molecules and not a true protein molecule. Hence the nitrogen precipitated by trichloroacetic acid, which was designated as the difference between total and nonprotein nitrogen, could well represent a polypeptide rather than a protein nitrogen.

Further evidence of the safety of this hydrolysate was obtained by the LD_{50} in mice. Accordingly, an average adult could possibly tolerate 57 Gm. of this material or up to 1,140 ml. of commercially available solution subcutaneously or intramuscularly. In addition, it was found that there was no evidence of hemolysis when human erythrocytes came in contact with 4.8% concentration of the hydrolysate.

The clinical evaluation of this product will be the subject of a subsequent paper.

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Determination of Dextrosulphenidol

By ARLINGTON A. FORIST and SUSAN THEAL MADDEN

DEXTROSULPHENIDOL, D*d* - threo - 2 - dichloroacetamido - 1 - (4 - methylsulfonylphenyl) - 1,3-propanediol, is a potent antibacterial agent. Early assays of this compound were based on a tube dilution technique (1). Recently, a chemical method based on alkaline hydrolysis followed by periodate oxidation and colorimetric determination of the resulting *p*-methylsulfonylbenzaldehyde has been described (2). The present procedure provides an alternative, somewhat simpler, analysis for dextrosulphenidol in the bulk state and in formulations, based on the determination of dichloroacetohydroxamic acid produced on reaction with alkaline hydroxylamine.

EXPERIMENTAL

Reagents.—(*a*) Hydroxylamine hydrochloride, 12.5% in methanol. (*b*) Sodium hydroxide, 12.5%, in 85% methanol. A sample of 12.5 Gm. of sodium hydroxide is dissolved in 15 ml. of water and the resulting solution is cooled and diluted to 100 ml. with methanol. (*c*) Ferric perchlorate, stock solution. An 800-mg. portion of iron powder is mixed with 3 ml. of water. A total of 10 ml. of 70% perchloric acid and 7 ml. of water is slowly added, dropwise. The resulting solution is then carefully heated to dissolve any remaining iron particles and to insure conversion to the ferric state. The cooled solution is quantitatively transferred to a 100-ml. volumetric flask and, with cooling under running tap water, gradually diluted to 100 ml. with anhydrous 2B ethanol. (*d*) Ferric perchlorate, reagent solution. A 40-ml. aliquot of the stock solution is transferred to a 1-L. volumetric flask followed by 12 ml. of 70% perchloric acid. The resulting solution is gradually diluted to 1 L. with anhydrous 2B ethanol with cooling under running tap water. (*e*) Alkaline hydroxylamine reagent. Equal volumes of reagents (*a*) and (*b*) are mixed and the insoluble sodium chloride is removed by filtration through Whatman No. 40 paper. This reagent is usable for four hours. (*f*) Standard dextrosulphenidol solution. An approximately 0.008 *M* solution is prepared by dissolving an accurately weighed sample in an appropriate volume of anhydrous 2B ethanol.

Procedure.—A sample of approximately 45 mg. of the material to be analyzed is accurately weighed into a 25-ml. volumetric flask, dissolved in anhydrous 2B ethanol, and diluted to volume. A 5-ml. aliquot of this sample solution is transferred to a 50-ml. volumetric flask followed by 3 ml. of the alkaline hydroxylamine reagent. The solution is mixed and the tightly stoppered flask is immersed in a 50° constant temperature water bath for forty-five minutes. At the end of that time, the solution is diluted to about 40 ml. with the ferric perchlorate reagent, thoroughly mixed, and stored in the

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absence of light for about ten minutes. (Usually several samples are run simultaneously and each is brought to this stage before proceeding further.) The solution is then diluted to 50 ml. with the ferric perchlorate reagent and thoroughly mixed. The absorbance of the resulting solution is measured within one hour at 530 mμ *versus* a reagent blank similarly prepared (Beckman model B spectrophotometer, 1-cm. cells).

Two standard samples are run in parallel with the unknown, one containing 5 ml. of the standard dextrosulphenidol solution, the other containing 2 ml. of the standard solution and 3 ml. of anhydrous 2B ethanol. Dextrosulphenidol content of the unknown is calculated from a linear standard curve constructed from these two points and the origin.

RESULTS AND DISCUSSION

The reaction of amides with alkaline hydroxylamine to produce hydroxamic acids which in turn form highly colored ferric complexes has been described by several investigators (3–9). However, under the conditions employed, instability of the ferric hydroxamates presented a serious problem. Recently, Goddu, LeBlanc, and Wright (10) have made a complete study of the factors involved in the hydroxamic acid procedure for the determination of esters and have described concentrations of acid and ferric ion necessary for a stable color. These conditions have been incorporated into the present method for the determination of the amide group of dextrosulphenidol.

Formation of dichloroacetohydroxamic acid at 25 and 50° is shown in Fig. 1. Reaction at 25° is incomplete after sixty minutes whereas at 50° maximal response is obtained after thirty minutes. The hydroxamic acid is stable in the reaction mixture for at least an additional thirty minutes.

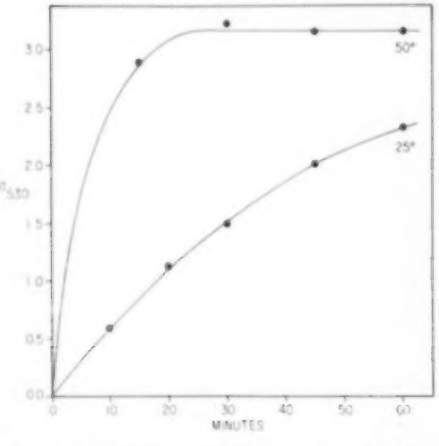

Fig. 1.—Production of hydroxamic acid from dextrosulphenidol.

Table I.—Determination of Standard Dextrosulphenidol Samples

Taken, mg./ml.	Found, mg./ml.	Recovery, %
0.52	0.53	101.9
1.04	1.08	103.8
1.04	1.04	100.0
1.56	1.59	101.9
1.56	1.62	103.8
2.08	2.05	98.6
2.08	2.02	97.1
2.08	2.01	96.6
Mean ± standard deviation		100.5 ± 2.8

Under the conditions employed the color is stable for at least an hour in the absence of light. To avoid photochemical instability samples should be protected from direct light as much as possible. Absorbance at 530 mμ follows Beer's law over the range 0.2 to 2.2 mg. of dextrosulphenidol per ml. of sample solution. The response varies slightly from day to day and for maximum accuracy standards should be run in parallel with unknown samples.

Representative data obtained in the analysis of standard dextrosulphenidol solutions (Table I) show a mean recovery of 100.5% with a standard deviation of ±2.8%. The method is rapid, accurate, and precise, and is specific for intact dextrosulphenidol in the presence of its hydrolysis products.

Other functional groups capable of yielding hydroxamic acids under the conditions employed, such as esters (11), imides (12), anhydrides, acid chlorides, and lactones, constitute positive interferences. High concentrations of carbonyls, transition elements, and ions capable of forming complexes with ferric iron will affect the intensity of the color and should be avoided.

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Steroids VI. Thiazoloandrostanes

Sir:

The recent report (1) of the synthesis of two thiazoloandrostanes, thiazolo-[d-3,2]-17α-methyl-5α-androst-2-en-17β-ol (II) and 2′-methylthiazolo[d-3,2]-17α-methyl-5α-androst-2 - en - 17β - ol (III), and the disclosure that III is a highly potent anabolic agent possessing only low androgenic action, prompts us to describe some of our work in this field. We wish to report the synthesis of five thiazoloandrostanes including the two reported thiazoloandrostanes, II and III.

17α-Methyl-5α-androstan-17β-ol-3-one was treated with pyridine hydrobromide perbromide in tetrahydrofuran to obtain 2α-bromo-17α-methyl-5α-androstan-17β-ol-3-one (I), m. p. 185–187°[1] (reported m. p. 196–198°) (1).

Anal.—Calcd. for $C_{20}H_{31}BrO_2$: C, 62.66; H, 8.15; Br, 20.84. Found: C, 62.54; H, 8.14; Br, 21.30.

The 2α-configuration of the bromo group was established by the hypsochromic shift of the C=O stretching from 5.85 μ to 5.76 μ upon bromination (2).

The bromoketone (I) was treated with thioformamide, thioacetamide, thiourea, N-allylthiourea, and N-phenylthiourea to prepare the following thiazoloandrostanes,[2] respectively: Thiazolo-[d-3,2]-17α-methyl-5α-androst-2-en-17β-ol (II); m. p. 177–179°; $[\alpha]_D$ +48.8°; $\lambda_{max.}^{EtOH}$ 251 mμ, log ϵ 3.64; $\lambda_{max.}^{CHCl_3}$ 2.75, 3.00–3.05, and 6.40 μ (reported: m. p. 192–193°; $[\alpha]_D$ +33°; $\lambda_{max.}^{EtOH}$ 252 mμ, log ϵ 3.61) (1).

Anal.—Calcd. for $C_{21}H_{31}NOS$: C, 73.02; H, 9.04; S, 9.28. Found: C, 72.66; H, 9.01; S, 9.53.

2′ - Methylthiazolo[d - 3,2] - 17α - methyl-5α-androst-2-en-17β-ol (III); m. p. 198–199.5°; $[\alpha]_D$ +35.6°; $\lambda_{max.}^{EtOH}$ 253 mμ, log ϵ 3.72; $\lambda_{max.}^{CHCl_3}$ 2.76 and 6.40 μ (reported: m. p. 212–215°; $[\alpha]_D$ +47°; $\lambda_{max.}^{EtOH}$ 254 mμ, log ϵ 3.76) (1).

Anal.—Calcd. for $C_{22}H_{33}NOS$: C, 73.48; H, 9.25; N, 3.90; S, 8.92. Found: C, 73.42; H, 9.23; N, 4.12; S, 9.04.

2′ - Aminothiazolo[d - 3,2] - 17α - methyl - 5α-androst-2-en-17β-ol (IV); m. p. 270–272°; $[\alpha]_D$ +45.0°; $\lambda_{max.}^{CHCl_3}$ 2.76, 2.86, 2.94, 6.25, and 6.57 μ.

Anal.—Calcd. for $C_{21}H_{32}N_2OS$: C, 69.95; H, 8.95; N, 7.77. Found: C, 69.80; H, 9.26; N, 7.47.

2′ - Allylaminothiazolo[d - 3,2] - 17α - methyl-5α-androst-2-en-17β-ol (V); m. p. 232–234°; $[\alpha]_D$ +50.5°; $\lambda_{max.}^{EtOH}$ 266 mμ, log ϵ 3.93; $\lambda_{max.}^{CHCl_3}$ 2.76, 2.90, 6.07, 6.27, and 6.49 μ.

Anal.—Calcd. for $C_{24}H_{36}N_2OS$: C, 71.96; H, 9.06; N, 6.99. Found: C, 71.96; H, 9.13; N, 7.15.

2′ - Phenylaminothiazolo[d - 3,2] - 17α-methyl-5α-androst-2-en-17β-ol (VI); m. p. 250–252°; $[\alpha]_D$ +46.0°; $\lambda_{max.}^{EtOH}$ 298 mμ, log ϵ 4.21; $\lambda_{max.}^{KBr}$ 2.76, 2.90, 3.06, 6.19, 6.48, and 6.64 μ.

Anal.—Calcd. for $C_{27}H_{36}N_2OS$: C, 74.27; H, 8.31; N, 6.42. Found: C, 74.61; H, 8.41; N, 6.14.

The paper (1) which reports the preparation of structures II and III contains some errors, e. g., the use of nomenclature which describes the fusion of the thiazole ring as b-3,2 rather than d-3,2 indicates that fusion occurs between positions 2 and 3 of the thiazole ring. The ring is fused between positions 4 and 5. Our rotation data on II and III do not agree with the reported data (1). Perhaps the reported data on these compounds have been interchanged. The melting points reported (1) for I, II, and III are 11–15° higher than ours. We have rechecked the thermometer and melting points and cannot account for these differences.

We had prepared these thiazoloandrostanes as potential anabolic agents following the lead provided by Sterling-Winthrop Research Institute when they announced the high anabolic/androgenic ratio of 17β-hydroxy-17α-methyl-5α-androstano[3,2-c]-pyrazole (3). The products described in this paper were submitted to Smith Kline and French Laboratories for screening.

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Norman J. Doorenbos
Conrad P. Dorn, Jr.

Pharmaceutical Chemistry Dept.
School of Pharmacy, University of Maryland
Baltimore 1, Md.

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[1] Melting points are uncorrected and rotations were determined in chloroform.

[2] A similar procedure has been used for the preparation of 2′-aminothiazolo[d-3,2]-5α-cholest-2-ene. Antaki, H., and Petrow, V. J., *J. Chem. Soc.*, **1951**, 901.

Assay for Bithionol in Liquid Soap

Sir:

The method for the assay for bithionol in liquid soaps as recently described by Matuszak and co-workers (1) will undoubtedly give results which are slightly more accurate than those obtained by the method previously proposed by us (2).

We feel, however, that the following comments should be noted:

(*a*) The changes we introduced into the method of Childs and Parks (3), which made possible a uniform method of assay for liquid and nonliquid soaps (as well as other cosmetics), have been negated by the changes proposed by Matuszak, *et al.*, so that the method is again limited to liquid soaps, just like the original method of Childs and Parks.

(*b*) A second, and in our opinion more serious objection, relates to the accuracy which can be achieved. This is based on the fact that the soap-base of the bithionol soap (without the bithionol) has to be available. This limits the use of the method to the laboratory of the manufacturer concerned. In Europe, conditions in the trade of drugs and cosmetics are such that it is most difficult, if not impossible, to obtain from the manufacturer a dummy, blank, or control sample, of any product which a third party wishes to analyze independently.

(*c*) Matuszak, *et al.*, conclude in their summary that the buffer solutions used by us are no longer required. Yet they have to add, of course, a solution which brings the pH to 2. Moreover, addition of an alkaline buffer will be necessary if the soap does not have a pH of 8 of itself; e. g., the so-called "artificial soaps."

These considerations lead us to conclude, therefore, that the value of the changes proposed by Matuszak, *et al.*, are more theoretical than practical.

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H. J. VAN DER POL

N. V. Koninklijke Pharmaceutische Fabrieken v/h
Brocades-Stheeman en Pharmacia
P. O. Box 48
Amsterdam C, Holland

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